REVIEW ARTICLE

PROBLEMS IN THE EVALUATION OF DRUGS IN MAN*

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It is a standard cliché in the teaching of therapeutics, but it is nevertheless a fact, that every treatment should be a fresh experiment in therapy. The details of the clinical experiment and how it must be conducted to elucidate what it proposes to find out is, nevertheless, not ordinarily a part of our medical curriculum. However, therapeutics has now progressed to a point where the issues of what comprises the clinical experiment is a practical, indeed, a vital matter.

The reality and immediacy of this problem was brought home to us in New York when it was reported that 5 per cent of the patients in one of our outstanding teaching hospitals was there in consequence of a reaction to medication[1–7].

I find it difficult to plunge further into this argument without appearing to say that, despite the high quality of the technical knowledge and training which makes up modern medical education, I seem to think that physicians sometimes cannot tell whether they are helping patients by means of the drugs they give them or instead by some highly personal communication they make to the patient or that an apparent improvement is merely a chance development[8,9].

I am assuming that here, as in the United States, a physician's training provides him with a substantial background in the basic and clinical sciences and a well-developed skill in patient examination, and that the combination enables him to discover what is wrong with his patient, to decide whether his patient is getting better or worse, and to make a shrewd guess as to the outcome of the case. What is quite another matter, is that he probably has neither been taught nor encouraged to discover precisely why, as a consequence of all the factors which enter into the complex that make up the physician's ministrations, the patient does get better or does get worse. By this I mean to imply more specifically that the physician, who learned as a medical student to determine whether and how a drug raised or lowered blood pressure in a cat, is usually not prepared to say after the administration to his patient of the drug whose action in the cat is so well documented, whether his patient's blood pressure rose or fell or refused to do either as a consequence of the medication, the medicating, the medicator or as a result of some other circumstance. This, however, is the basic problem in the clinical evaluation of drugs, one which is seriously neglected in the training of the medical student.

* *Based on a Special University of London lecture given at King's College, Strand, April* 30, 1959.

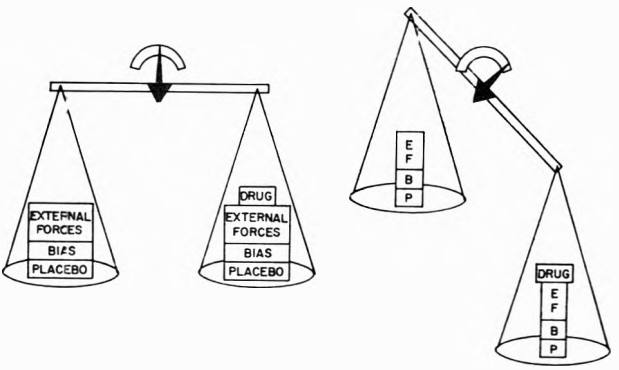
Nor is he taught how to examine a publication on therapy critically. Yet publication of a fallacious, poorly documented or inadequately analysed statement is almost always unfortunate for, regardless of its truth or substance, by virtue of publication alone, a medical statement, even a Letter to the Editor, acquires authority and, what is even more likely to lead to trouble is, that while it is relatively easy to have it published, it is far more difficult to erase a published blunder or delusion from the minds and memory of the medical profession, especially if it is a hopeful one. There are serious implications here, for regardless of what their real merit eventually turns out to be, drug manufacturers will continue to advertise the laudatory published statements long after substantial evidence to the contrary is adduced, to the confusion of the physician who tries to understand what is going on and to the misfortune of the patients of the physicians who do not. Unless the physician himself has some knowledge of the standards by which drugs may be properly evaluated and of the fundamentals of good design in drug evaluation, only the always well-intentioned but, alas, sometimes fallible, journal editor stands between him and reliance on shaky or spurious claims.

Criteria for separating substantial claims from the insubstantial must be set and methods of drug evaluation must be developed which not only differentiate between the good and the bad, but which also distinguish between the good and the better. This is not to say that there are yet no high standards and no good methods, for we have both, but the necessity for using them is not always recognised, and sometimes they are misused—viz, the large number of papers on new drugs containing claims which soon prove to be meretricious.

I would like to pursue, therefore, an examination of the many factors which influence patient response after the administration of a drug and which make it difficult to determine why he reacts as he does, and how one goes about the business of distinguishing between an alteration in the patient's physiologic state as a consequence of the direct effect of a medicament and as a consequence of one of the many influences in everyday life which impinge on physiologic function and which are generally briefly disposed of by our calling them chance occurrences. These must be distinguished if one is to know whether a particular medication is any better, or worse, than a simple lactose tablet.

The issues for resolution in the clinical evaluation of drugs are basically the same as for well-designed experiments in all other experimental disciplines and can be stated simply enough: identification and control of all the factors which may interfere with or assist in making observations and in collecting and evaluating data. This is what I should like to consider here in outlining the problems in clinical evaluation. For this purpose I propose to use as a model, scales which weight the evidence for and against drug action. In a proper clinical evaluation the effects of drugs *per se* are matched against all other influences which tend either to prevent the action of the drug from swinging the balance in the proper direction or to tip it in the other direction and simulate an expression of

drug action and, in either case, to provide answers which are not spurious and due to some other active force, and which otherwise may be misinterpreted as being evidence of action or lack of action of the drug[10–18].



FACTORS WHICH INFLUENCE DATA IN CLINICAL EVALUATIONS

The factors which influence data in clinical evaluations, especially when the effects of drugs on subjective responses are involved, may be enumerated as follows: (1) the pharmacodynamic action, (2) the dosage, (3) the subject, (4) the controls, (5) placebo actions, (6) bias, (7) the forces extraneous to the experiment, (8) the collection of data and (9) the sensitivity of the method.

Pharmacodynamic Action

When objective measurement of effects is possible, pharmacodynamic actions present the least difficulty in their evaluation. When pharmacodynamic actions are potent, reproducible, and are not significantly influenced by psychic forces, evaluation is also relatively simple. The action of a mercurial diuretic, for example, lends itself to dependable and relatively precise measurement and a true bioassay may be relatively easily performed in man because a measureable loss of weight, due to the effect on the drug on oedema, can be precisely translated into diuresis, an action which usually is not appreciably compromised by external forces[19,20].

Drug actions which must be evaluated in terms of subjective responses, and especially those which are not in themselves impressive, are far more difficult to evaluate. Thus it is exceedingly difficult to demonstrate differences in the analgesic effect of drugs of the order of effectiveness of aspirin, while it is simple to prove that morphine is effective, and still easier to prove that general anaesthetics are even more potent pain-relieving agents[15,21,22].

The measurement of the useful actions of hypotensive drugs is difficult, even though blood pressure can be measured precisely, because of the tendency to wide spontaneous variation in blood pressure in patients with hypertension as well as because blood pressure is also readily altered by immediate circumstances, tension, position, strain and rest[23,24].

Each drug, therefore, requires careful consideration with respect to the method most appropriate to the observation and measurement of its action[25,30].

Dosage

The proper evaluation of drug action requires the use of the proper dosage. It is obvious enough that when dosage is too low, regardless of the pharmacodynamic actions or potency of the drug, clinical evaluation will not reveal any difference between the drug and a placebo, and when the dosage is too high any therapeutic effect will be obscured by the toxic effect. This is why we have so little precise and dependable information about the actual usefulness of the tranquillisers in the treatment of the everyday simple anxieties that we all suffer, while there is substantial information about their value in the treatment of schizophrenia. In the latter condition large enough doses are used to observe and estimate or actually measure effects. In the former we use token doses and hope for subliminal effects. These of course defy ordinary methods of estimation or measurement and, as a result, we not only do not know which tranquilliser is better for the run-of-the-mill anxiety but we also do not really know whether many are any good at all[28].

Neither toxic nor token dosage may be used in clinical evaluation; dosage must be carefully chosen. If a single dose is used it is usually preferable that it be one which, by preliminary examination, is found to be on the sensitive portion of the dosage-response curve of the drug. There are some designs which use threshold or ceiling doses, and although these often appear to have practical as well as logical advantages, a serious disadvantage lies in the fact that effects on the extremes of dosage-response curves are difficult, sometimes impossible, to evaluate precisely. The best way to compare drugs is to use a series of graded doses of each. This provides a more substantial basis for comparison than that of single doses, no matter how well-chosen the latter may be, for it also serves as a sort of built-in measure of the sensitivity and the discriminating powers of the method—an internal operational control.

The Subject

In much the same way that some species of laboratory animals are superior to others for particular experiments in the laboratory the choice of a suitable subject is often a critical matter for an investigation in man. Thus, while the best subject will tend to make the method more sensitive, unsuitable subjects may make the method so insensitive that it is unable to detect the particular drug action under investigation and, therefore, regardless of the effectiveness of the drug, provides only negative answers.

The argument that the patient with the disease for which the drug is ultimately intended is the best subject for the evaluation of the drug is not always correct. When he is the only possible subject, he is, perforce, the best subject, but there are also situations in which there is a choice, and sometimes he may be unsuitable. The choice rests first of all on the purpose of the investigation, whether it is to define the action

of a drug in man, that is to say, a pharmacologic examination or to predict the value of a drug in the treatment of a particular disease, that is, a therapeutic evaluation. These have fundamentally different goals and it may not be assumed or even implied that the results of one type of investigation invariably applies to the other.

Some amplification is necessary. The investigation of the pharmacodynamic actions of a drug in man is a necessary preliminary to its therapeutic evaluation. Although the first may have implications of utility in particular disease states, indeed provide clues to therapeutic usefulness which are ultimately borne out by subsequent experience, sometimes the clinical trial of the drug fails to confirm the suggestive pharmacologic findings in man as well as in animals. Basic pharmacologic information on the effects of the drug in man is essential for its therapeutic exploration but only the therapeutic evaluation of the drug in the patient with the disease will provide the conclusive evidence of its value in that disease. Each approach serves a particular and indispensable purpose in the final evaluation of the therapeutic potential of the drug, but only rarely can both be carried out simultaneously. The therapeutic evaluation of a drug cannot, therefore, be carried out reliably in any subjects but those for whom its use is proposed. And if the evaluation is to have predictive value, the group of subjects must represent a fair, or random, sample of the patients who suffer from the disease.

But, the problem in the choice of subjects for the exploration or evaluation of the pharmacodynamic actions of a drug in man is a fundamentally different one[31–35]. Since the disease state *per se* is not being examined here, in so far as it is possible, the complex of factors which make up the disease, as well as all the other known and unknown factors which influence and which may obscure the examination, measurement and evaluation of changes in man's functional state as the result of drugs, must be identified and eliminated or controlled in some way.

Whereas the utility of a diuretic in the treatment of congestive failure can be predicted only after an experience with a representative sample of patients with congestive failure, the effects of diuretics on particular electrolytes, or the influence of electrolyte load on diuretic action can be much better explored in healthy subjects. This is not to say that the normal subject is always the best for pharmacological investigation. There are situations, for example, the effect of a drug on cardiac arrhythmias which demand the afflicted patient for the pharmacologic as well as the therapeutic exploration. There are also situations in which there appears to be a choice. For example, drugs for motion sickness may be investigated in voyagers aboard ship or in healthy subjects using the Barany test[36–38]. Additional examples will not further clarify the idea that, in the exploration of the pharmacologic action of drugs in man, there are drugs which are so highly specific that only the patient with the disease for which the drug is intended can be the subject, and there are drugs for which both the patient and the normal man are suitable subjects for evaluation, in which case the choice may be based on convenience alone, and finally, there are drugs for which the normal man appears

to be clearly the more desirable subject for pharmacologic evaluation than the patient.

Subjects must be selected in a manner which insures the ability of the group as a whole to discriminate between "active" and "inert" agents; that is to say that the subjects must be sufficiently sensitive to the drug action under investigation to be able to appreciate differences of practical significance. While it is usually desirable that the group of subjects be a representative one, above all it must be sensitive in the sense that it must be able to detect an effect should it develop. Where large numbers do not provide this by chance alone, efforts may have to be made to impart this quality to the group by deliberate elimination and selection.

The question may arise whether to use in-patients or out-patients as subjects for a particular study. The former provide the advantages which ensue from a relatively protected and more or less constant environment while the latter provide the advantage of large numbers at low cost as well as little work in patient care. However, there are other features which bear on the choice of one over the other which are not so apparent. The patient in the hospital has little incentive to physical or mental activity and, as a matter of fact, he is likely to spend a great deal of this time in bed and to be especially pleased if he can escape some of the ennui of his hospital stay by napping a large part of the day as well as sleeping through the entire night. The out-patient, on the other hand, is far more active and, through exposure to the usual activities of living, is regularly challenged throughout the day by one stress or another. I have observed that the latter patients appear to appreciate the actions of sedative drugs more than the former and, I am inclined to believe that this may be due to the difference in the daily living experience and frequency of stress challenge in each case. This difference may also be important in other areas of drug evaluation.

Subjects likely to give misleading results must not overwhelm the group. Where sex makes a difference, the group may be selected accordingly. That dosage-response may be unusual in the very young must sometimes be taken into account. The potentially high side-effect liability of patients with renal disease, and the elderly in general, must be considered. In studies involving subjective criteria, exceedingly phlegmatic subjects desensitise the method by not reacting, while exceedingly neurotic and over-reactive or suggestable patients tend to compromise the sensitivity of the method through wide swings of mood and attitude as the result of placebo as well as of active medication. In no event should the number of unusual, abnormal, or resistant subjects be excessive and, there are suggestions in the literature that, given the proper basis for their elimination and, with the standards established at the outset, any or all such may properly be removed before the study is begun.

Knowledge of participation in the exploration or any kind of special examination of a new drug, to say nothing of the highly charged concept of being the subject of an experiment, seems to exert special psychic pressure on the subject and make him act in something other than his usual manner, to be overly introspective, to try to help the investigator

or, in some instances, to react with fear or resentment. Some patients will tolerate discomforts with less difficulty when they know they are participating in an investigation; on the other hand, some may be less tolerant. But however they may react, information that they are participating in an experiment alters the subject and, thereby, adds another element to patient reaction and, in that way, desensitises the method. In some trials it is not possible to keep the fact from the subject but, generally, the optimal subject is one who does not know that he is participating in an experiment[13].

This leads to the important practical problem of the acquisition of subjects for studies, always an important and often a difficult and limiting one, especially when large groups are necessary. It would seem at first that the most reasonable and just method is to call on volunteers. But the volunteer is not a normal subject; he is a *volunteer*, and he may present the problems just cited based on this fact. In addition, the highly co-operative and willing volunteer may provide a set of highly personal psychological problems. In an interesting analysis of this problem by Lasagna and Von Felsinger, it was shown that the volunteer is an unusual subject and often clearly undesirable on that account[39].

Collecting an indiscriminate group of subjects for drug evaluation and dealing with the problems of chance differences in subjects merely by the process of randomisation simply equalises the influence of a large amount of dead weight for, in order to overcome the spurious swinging of our balance, an equal number of unsuitable subjects are put on both pans. However the choice of the subject is finally made, it should be made with the idea in mind that his proper selection has a great deal to do with the ultimate sensitivity of the method.

The Controls

In no discipline can an experiment be pursued without a control. Even those experiments which the experimenters, themselves, presume to be without controls, are nevertheless, controlled. The control is the only basis for a comparison and, thus, there is a control implicit in every judgment on a drug. Somehow or other the statement is made or implied that one drug is better or worse or equal to some other drug or to no treatment at all. The only important question about the control is, therefore, not whether one has been used, but rather whether it is sound as a basis for the comparison on which the judgment is based.

It is tempting to use the easy way out; to use what has been called the historical control, that is to say, a recounting of previous personal or recorded experience as a basis of comparison. Often, this is not recognised as a control—and understandably so—for it is a treacherous one. No method of drug examination is more likely to lead to erroneous conclusions. It has none of the safeguards provided by other controls, the elimination of placebo effects and of bias and the natural course of events and chance as the effective forces in apparent drug action. It also fails utterly to provide comparable bases for examination of control and experimental groups. Only in the case of the disease in which an

irrevocable or unquestionably characteristic course has been established, and particularly when the condition is rare, is the historical control justified.

The classic experiment employs separate groups for control and treatment, but this provides significant data useful for statistical validation only when the groups are formed by random selection and when they are extremely large or extraordinarily homogeneous[40]. In man, this poses a serious practical problem. Since the human equivalent of litter-mates in the laboratory, that is, sets of identical twins, are too rare to be hoped for, proper matching of control and experimental groups is essential. When the number of subjects is sufficiently large and patients are put in either group by a process of random selection, chance alone will insure a proper balance. Matching of control and experimental human subjects by actual selection, however, is an insuperable task.

An alternate method which is acceptable, is to give each patient the medicaments and placebo serially so that each subject serves as his own control. This is the so-called cross-over design. When the condition of the patient during the control and the experimental periods is similar, if not identical, there is a valid basis for comparison. In clinical evaluations this plan is often the more satisfactory because smaller numbers are needed, hence the study can be conducted in less time and with less cost. On the other hand, when the subjects have progessive disease, it may be inappropriate to compare the effects at two stages of the disease and, in such a case, only separate control and treated groups are acceptable. Another difficulty with the second plan is that each subject is required to participate in the entire course of the experiment and, in the usual study, a discouragingly large percentage often fail to do so, thereby increasing the number of original subjects necessary for the study and necessitating a design which will not collapse if a subject defaults.

Placebo Actions

The term "placebo" has taken on many implications not within the philologic meaning of the word, as for example, "negative placebo actions". As the word is currently used in clinical evaluations, it includes a large series of visceral, somatic and psychic responses to the physician, to his presence, to his words, to his ministrations, and to his medications. Such an action is inherent in all medications regardless of whether they are useful, hazardous, impotent, inert, unpleasant, inadequate, or inappropriate or for that matter, new or old, as long as the medication is prescribed by the physician himself. To be certain that these are not the only effects of drugs under examination, it is essential to have a basis of comparison of drug effect with "pure" placebo effect. To provide this, one must also give an inert medication which is otherwise identical with the drug under examination. It has been suggested by Gaddum that such an inert material is more properly called "dummy" than "placebo"[41]. But, "dummy" or "placebo", an inert control medicament must be given in all clinical studies to distinguish between the effects of the *act* of drug

administration and the pharmacodynamic effects of the medicament itself[42–52].

Such a measure provides the only defence against the suggestion that results reported after the administration of a drug are due to placebo actions rather than to the pharmacodynamic action of the drug itself. In using placebo for control it is well to recognise that in the analogy provided by our chemical balance the placebo is not restricted to one pan and the drug action to the other. Since placebo action is inherent in every act of medicating by the physician there is, in fact, placebo in both pans, and the scales merely measure the difference between them. That is to say, placebo effect is being exerted on both pans at all times and the only measurement is of that which the drug may provide in excess of its inherent placebo action and, in the event that the two do not summate, it measures merely drug action which is not masked by placebo action.

Bias

In addition to the considerable psychic force exerted by the administrator of a drug if he be an accredited member of the medical profession, the so-called placebo action of drugs, the hopes of the patient and the therapist alike, as well as any bias either may have with respect to treatment or experiment, also exert considerable force on patient response after the administration of drugs and, therefore, on the art of the collection of the data. Therefore, these must also be reckoned with in all clinical evaluations.

The patient may want to get better to the extent that he is inclined to see good effects after administration of any new medication, and colour his subjective responses accordingly. On the other hand, he may find compensations in his illness and wish to preserve his complaints, hence be inclined to depreciate pharmacodynamic effects, sometimes miscalled "negative" placebo action. The physician's knowledge of the nature of the medicament is exceedingly important, for regardless of how much he tries, if he knows the identity of the medicament, he may nonetheless relay this information to the patient. In addition, his understandable bias may lead him to interpret, hence modify, data along preconceived lines as he collects it and, as a result, there may be substantial apparent effects from accumulated bias. The importance of the unconscious communication of the physician was proved in a study in which patients could not detect the difference between placebo and aspirin unless the physician prescribing them himself knew which was which[53]. The standard procedure is not only to use placebo and drug which are identical in appearance, but also to keep both the physician and the subject ignorant of which is in use at the time of the prescribing, questioning, and examining.

Of all the devices to insure valid data, none seems to have attracted so much attention and to have evoked so much controversy as this so-called double-blind technique. It is a philosophically sound, as well as practical, control device to use in clinical evaluations to deal with the tendency of conscious and unconscious bias to obscure and distort the

effects of drugs. It deals with the influence of the physician's bias (his professional purpose to help his patient as well as his preconceived ideas and prejudices about the medication and his unconscious communication to his patient) on his observations by blinding him, that is, keeping him ignorant of whether he is giving or has given his patient placebo or active drug. So also are the effects of the patient's bias (his hopes and his anxieties) on his estimates of his subjective responses dealt with by blinding him, that is, keeping him ignorant of whether he is receiving or has received active drug or inert tablet of identical appearance; hence the double-blindness. What is important to remember in this connection is that the myopia and astigmatism of the physician and the subject due to bias are corrected only in the sense that blinding will compensate for them, and that nothing has been added to increase the visual acuity of either observer.

The question arises whether the double-blind control must invariaby be used in clinical evaluations. This has been reviewed by Gold[54–57]. Much as we favour its use, occasionally it does not seem feasible. There are instances in which the drug promptly reveals itself by its unmistakable side effects and automatically removes one or both blindfolds by an action other than the one for which the drug is being examined. How could one use the double-blind control in a study comparing a general anaesthetic and a placebo? Page and Corcoran point out that, although the physician may remain blind, with many hypotensive drugs the patient's blindness soon vanishes because the drugs have obvious effects in addition to the hypotensive action and, in such a case, only the physician remains blind[16,58]. Despite such difficulties, there are sometimes devices for circumventing them.

Perhaps because of its dramatic qualities, the double-blind technique has attracted widespread attention. It has also apparently been widely assumed that it is a complete method of evaluation it itself, instead of being only a control device. Indeed, it is often called the double-blind *test*. Many seem to believe that all that is necessary for a good clinical evaluation is to use the double-blind technique and, regardless of all other details, inevitably and automatically, the results obtained will be valid. Since it is relatively easy to apply the double-blind technique, some are using this as the only control measure in the design of their clinical evaluations. In many publications it is stated in the title itself that the double-blind control was used, not only as if the use of a control in an experiment was so exceptional as to be worthy of special mention, but also as if to indicate in advance of reading that a special type of insurance has been taken out to guarantee that the results about to be recounted were bound to be above reproach[53,59–63]. The enthusiasm for this catch-phrase has grown so that "triple-blind studies" have already been described and I read recently that "a five-way blind cross-over was carried out"[64]. It would seem that the fascinating notion is developing that if there is sufficient blindness it will ultimately lead to some sort of occult vision.

Unfortunately for those of us involved in clinical evaluation of drugs, the double-blind control does not provide either a simple or a complete

solution to our problems any more than a control is all that is needed by experimenters in any other discipline for the complete design of an experiment. Nor does it eliminate bias as an element in the method; it merely deals with it by equalising its effects so that, as weighed in our scales, unequally distributed bias alone will not account for the apparently decisive evidence.

Forces External to the Experiment

There are a large number of extraneous influences which affect the state of the subject's physical, functional and psychic state—a change in the course of his illness, a happy experience, a lost job, a family quarrel, a seasonal allergic state, a change in the weather, a turn in world affairs —which may also influence his response to drugs. That is to say, there are changes in the subject's state which develop after the administration of a drug and therefore may appear to be responses to drug action. These may be both objectively and subjectively recorded responses[65,66].

To a limited extent, these factors may be reduced by removing the patient from his home to the protective atmosphere and routine of the hospital where those influences that disturb are likely to remain relatively constant compared with the much more labile scene in the usual home under the best of circumstances. This is not always so, however; some patients may find the hospital environment disturbing rather than protective and restful and, for them, this change makes them poorer subjects for drug evaluation.

The tendency of external forces to influence response can be dealt with by prescribing medication and placebo by a scheme of random distribution so that the disturbing forces affect the apparent response to placebo and drug alike, and being spread equally they appear to favour neither. It is to be pointed out that, by this scheme, the influence of external events on the apparent response of patients to drugs is not eliminated, but is spread equally, that is, divided equally between both sides of the scales, so that the scales do not swing by virtue of extraneous forces alone. It is also to be noted that where it is possible to reduce these forces, for they can never be entirely eliminated, less is then placed in each pan, and, to that extent, the scales are less burdened with dead weight.

Collection of Data

When objective measurement is possible and differences can be expected to be large, there is relatively little difficulty in the collection of data. However, when the patient must communicate his subjective experience, it is quite another matter. There are few experimental procedures in which so vital a part of an experiment as the collection, storage and interpretation of observations is left in the hands of an interested, biased, and untrained assistant, yet this is precisely what is being done when the patient-subject is asked to report and summarise his experiences after a period of medication. How can he help having his total recollection affected more by recent events than those farther back in his memory.

What happened two days or two weeks ago, or even two hours before questioning can be coloured by the patient's annoyance over some action of the receptionist in the out-patient waiting room.

The daily report-card system was designed to deal with this defect in the interval report system by decreasing the interval between the recording of respones to one day[67]. When these records are kept by the patient himself, however, the improvement in methodology is more apparent than real. At the very best, it substitutes a 24-hour recall for, say, a two-week recall, but it still has the same fundamental deficiency. In practice, it is often no better than the longer interval system. I have observed patients filling out their "daily" report cards while sitting on the benches waiting to be called to turn in cards that were supposed to be filled out faithfully each night during the two-week interval between examinations. While this method of data harvesting obviously supplies more data than a longer interval report system, it has not been subjected to an analysis which proves that the data itself or the answers derived through its use are more substantial. Other improvements have been suggested; having the subject mail a postcard each night or telephoning a report each night, but even these compromises leaves the data subject to the caprice of the patient for too long[68].

Not so long ago, two of us separately examined the effects of aspirin in the relief of pain, each using a different method of collecting data, but in all other details, a similar design[15]. The drug and the doses were the same, there was the same use of placebo and double-blindness, randomisation, and so on. One of us used a two-week report card system while the other questioned the patient during the course of the action of the drug.

The first method provided a large number of cards, hence a large amount of data. Statistical analysis of the data provided by this method showed no significant difference between the analgesic effects of placebo and aspirin, hence the answer that aspirin was without effect on arthralgic pain. In the second method, the statistical analysis of the data not only indicated a significant difference between the analgesic action of aspirin and placebo, but it also described parameters for aspirin action, a fine dosage-response curve and a curve of action. The only difference between the two methods was that the second collected patient responses directly from the patient as the course of drug action developed while the first permitted the patient to keep the data in his possession, subject to all the events of life which affected him, until he communicated it to us at some later date.

In general, any device which leaves the discrimination of the reaction to drugs at the mercy of patient recall provides the setting for outside influences on, and tampering with, data. Every effort should be made to minimise the period between the experience with the drug and the recording and collecting of the data; the data should be taken out of the patient's hands as soon as possible and, thereby, kept as nearly as possible in its original form.

We may now consider the effect that the collection of data has on our balance. As we allow time to alter data through our control process of randomisation, we once more burden both sides of the balance with yet more dead weight, weight immaterial to the problem at hand, which is the weighing only of pharmacologic actions.

Sensitivity of the Method

As in methods for chemical analysis, every design for drug evaluation requires a demonstration that its sensitivity is appropriate for the distinction it chooses to make. A scale of sensitivity should indicate first, the ability of the method to detect the drug action *per se*, and second, the increments in effects which it can distinguish. Without the first, a negative answer cannot be defended, and without the second, a positive answer has no quantitative meaning.

A negative answer is valid only if it is demonstrated at the same time that the method can also appreciate the effects of a standard and similar drug. The ability of a method to discriminate increments in effects can be indicated by its capacity for dosage-response when a series of graded doses of the standard or experimental drug is used. Such a scale of sensitivity gives positive results quantitative meaning.

The internal control just described is not only essential to establish the propriety as well as the sensitivity of the method as such, but it may not be eliminated in further clinical evaluations with the same method (as can be done with impunity in some other disciplines, for example, methods of chemical analysis can usually be repeated many times without rechecking accuracy and sensitivity). The need for a continuing test of sensitivity comes not from instability of the method, but from variations in the population of subjects which, at one time or another, may make them more or less able to discriminate between "active" and "inert" agents.

Interpretation of the Data

The current literature has placed overwhelming emphasis on one item in the design of a proper method of clinical evaluation, the double-blind technique, and relatively little on all the others. Unless all means of control are considered and given their proper importance in the design of clinical evaluations, improper and erroneous conclusions will be drawn from data that are supplied by studies which use the double-blind control just as well as from those which do not.

Which way the scales, which we have used as the model for methods of drug evaluation, swing, that is, whether drug action *vis-à-vis* chance is favoured, depends, of course, on the relative weight in one or the other pan. Whether the swing is meaningful or misleading depends on whether the weight which swings it is due to a specific action of the drug or to any of a myriad of forces which influence man's behaviour and his mental, physical and visceral activity. When such a model is used, one way to prevent swings of the balance by factors other than the intrinsic pharmacodynamic action of the drug itself, is to accept and spread their influence equally on both sides of the balance, thereby causing no disturbance in

balance by their weight. Nothing more than this is accomplished by the control devices of placebo, double-blindness and randomisation; they merely prevent chance or biased swings of the balance in either direction.

What is rarely taken into account in clinical evaluations is how much weight is necessary to make the balance swing at all, that is, the basic sensitivity of the method. Whatever the original sensitivity of the balance, consider what is done with it in the usual design for clinical evaluation. Consider that the scales are not empty at the outset of the evaluation, merely in balance. We place equally on both pans, placebo action of drugs, bias, the influence of diverse extraneous factors such as weather political events, family stresses, and a number of other vagaries of human experience that tend to mould or alter man's functional state and his response to drugs. It is to be repeated, these are not removed as interferences; they are preserved and spread equally over both pans of the balance by the process of randomisation and by the control of double-blindness. The balance is thereby dead-weighted with a large amount of material which is foreign to the specific problem at hand. No matter how sensitive originally, such a procedure makes the balance less sensitive just as an analytical balance sensitive to a fraction of a milligram under usual conditions is no longer swung out of balance by milligrams when dead-weighted with several kilograms on each pan.

Ultimately, therefore, the sensitivity of a method of clinical evaluation is a function of the relative weight of the pharmacodynamic force under investigation and the weight of the nonessential interfering forces which are treated by equalising them; the greater the former with respect to the latter the more sensitive the method and, vice versa, when the latter becomes relatively heavier, the method becomes proportionately less sensitive. To the extent that dead-weighting grossly desensitises the scales, this process can lead to erroneous interpretations in the sense that it indicates no differences whenever it is used to weigh forces which it can no longer sense.

Of the disturbing factors already discussed, some are subject to choice and, in that sense, the disturbance may be eliminated. Thus it may be possible to choose the proper dosage range, the most sensitive subjects, and the appropriate control. Some factors which cannot be eliminated may be modified; the removal of the patient from the home to the constant environment in the hospital may reduce the external variables. The collection of data on the spot reduces the treachery of patient recall. Finally, there remain some disturbances which cannot be reduced, removed or modified; bias and placebo actions. For those which cannot be eliminated there is only the double-blind control and randomisation to spread the prejudicial factors equally.

In the studies with aspirin cited briefly above, the reason for the discrepancy in the results by the two methods used is to be found in their relative sensitivity; one method provided a false negative answer because, in order to prevent a false positive answer, interfering forces were dealt with only by the desensitising process of acceptance and balancing out, thereby becoming too insensitive for its task, whereas the second was

sufficiently sensitive and gave a precise positive answer because it had eliminated the interferences to a practical degree.

A great danger in interpreting clinical evaluations lies in failure to recognise the meaninglessness of the negative answer when the method is not sufficiently sensitive for the purpose. The failure to demonstrate statistically significant differences between drugs or treatments is frequently misinterpreted to mean that no real differences exist. However reasonable the latter may seem from the data, an assertion that the drug or treatment effects are identical is not easily proved. Statistical tests of significance merely tell us the likelihood that whatever differences are noted in the data are due to chance. Thus, when the differences are statistically significant we are assured that this is unlikely to be a chance occurrence, and we may then, with a measurable degree of confidence, rightly or wrongly (for the statistics themselves do not validate the basic data), ascribe the results to essential differences in the effects of the drugs. Differences which are statistically insignificant could result simply from an inadequate trial or from an insensitive method of evaluation which statistical analysis may not indicate.

It is well to remember that statistical analysis proves nothing about the original validity of the data—it is merely a device for establishing the betting odds on the reproducibility of the results obtained by the same method, the predictability of similar conclusions with future experience under the same conditions. Statistical prognostication is always based on the assumption that the data used were worthy of collection; statistical analysis of poor data is tantamount to attempting to make a silk purse out of a sow's ear. Only when the design provides built-in controls, showing an ability to discriminate meaningful effects or to show graded effects with graded doses of the drugs, can any valid inferences be drawn from negative results (that is to say, statistically insignificant diffrences or, if you will, significant indifferences).

It should be made clear also that, although statistical procedure presently seems to have assumed an especially prominent position in reports on drugs, fundamentally this is not at all new. As with the use of controls, no matter how an experiment is planned, how the terminology seems to intrude, or how the results are expressed, statistical analysis is inseparable from clinical evaluation of drugs. It is a biologic fact that all physiologic reactions and failures to react exhibit some degree of individual variability and, as a consequence, any statement about the pharmacologic or therapeutic action of a drug has implicit in it the statement that this is not a chance occurrence. It is, therefore, a statement based on either a calculation or a guess of statistical significance; the only question which remains is its quality and its applicability.

However the experiment is designed, if the signicance of differences that are indicated by the data is to be established with a degree of assurance, the data must almost certainly be subjected to statistical analysis.

It is good practice, therefore, to plan the collection of data in such a way as to simplify subsequent analysis and interpretation[69–79]. This is not to say that the mere statement of statistical significance insures

correctness of their interpretation. If the data are inappropriate or improperly collected, as illustrated by the results of the study with aspirin, despite their statistical significance their interpretation may be erroneous.

Conclusions

Clinical evaluations are so beset by external disturbing forces that every possible control measure must be applied if valid and durable results are to be obtained. It has been pointed out that the selection of the proper dosage range is vital and that the selection of the proper subject is equally critical in the design for clinical evaluation. As far as possible all external disturbances must be eliminated. Data must be collected promptly and before any tampering has occurred. Treatments must be randomised. In addition to the use of the placebo control, the double-blind control should also be used whenever and wherever it is feasible. There is no conceivable disadvantage in the application of the double-blind control, only protection against spurious data, but it must not be used as a means of avoiding the elimination of bias and interfering psychic factors. I would like to emphasise as strongly as possible that its use will not validate otherwise poorly designed experiments. While it will prevent false positive interpretations, used in a poorly designed experiment, it will not prevent a false negative interpretation.

Each clinical evaluation must be sensitive enough to detect what it proposes to discover, and each experimental design must have built into it an indicator that it is capable of such detection. A negative conclusion is without merit unless there is incorporated in the clinical evaluation a demonstration that the method is competent to indicate a positive effect when it is present, i.e., an internal control. It is suggested that in clinical evaluations another, demonstrably effective, drug always be used in addition to the placebo control, to indicate this essential competence of the method.

Beyond this, there is the problem of the sensitivity of the method, the increments in effect which it can distinguish. Few clinical evaluations indicate what differences in effect they can discriminate. Yet in evaluations in all other disciplines, it is standard procedure to provide such a scale. Clinical evaluations cannot escape this requirement; the complete clinical evaluation must include a built-in sensitivity scale, and, through the use of graded doses, a demonstration of the increments in pharmacodynamic effect which the method can distinguish. When differences between standard and unknown or placebo are indicated, the sensitivity of the method to distinguish differences is thereby at hand to indicate the quantitative significance of the differences.

The definition of the effects of many drugs and the proof of the superiority of one drug over another require investigational designs which are based not only on the principles laid down here but which are also designed with due regard to the particular drug, the particular subject, and the particular circumstance under which they must be conducted. There is yet no standard method—there are basic requisities, essential controls, and some well-established procedures—but each different

pharmacodynamic action of a drug may need a different subject, a different control, a different circumstance, or a different design for its proper evaluation.

References

1. Barr, *J. Amer. med. Ass.*, 1955, **159**, 1452.
2. Friend and McLemore, *New Engl. J. Med.*, 1956, **254**, 1223.
3. Kirsner, *Ann. int. Med.*, 1957, **47**, 666.
4. Moser, *New Engl. J. Med.*, 1956, **255**, 606.
5. Rising, *Postgrad. Med.*, 1958, **24**, 200.
6. Schiffrin, *ibid.*, 1958, **24**, 305.
7. Wooley, *Perspectives in Biol. and Med.*, 1958, **1**, 174.
8. Coleman, Menzal and Katz, *J. chron. Dis.*, 1959, **9**, 1.
9. Dowling, *Arch. intern. Med.*, 1957, **100**, 529.
10. Goodwin and Rose, *J. Pharm. Pharmacol.*, 1958, **10**, Suppl., *24T*.
11. Lasagna and Meier, *Ann. Rev. Med.*, 1958, **9**, 347.
12. Menzel, Coleman and Katz, *J. chron. Dis.*, 1959, **9**, 20.
13. Modell, from *The Relief of Symptoms*, Saunders, Philadelphia, 1955.
14. Modell, from *Drugs of Choice*, C. V. Mosby Company, St. Louis, 1958.
15. Modell and Houde, *J. Amer. med. Ass.*, 1958, **167**, 2190.
16. Pannekoek, *Postgrad. med. J.*, 1957, **33**, 396.
17. Sabshin and Ramot, *Arch. Neurol. and Psych.*, 1956, **75**, 362.
18. Travell, *Amer. J. phys. Med.*, 1955, **34**, 129.
19. Modell, *Ann. int. Med.*, 1944, **20**, 265.
20. Modell, Gold and Clarke, *J. Pharmacol.*, 1945, **84**, 286.
21. Houde and Wallenstein, *J. Amer. geriatrics Soc.*, 1956, **4**, 167.
22. Wallenstein and Houde, *Fed. Proc.*, 1953, **12**, 377.
23. Murphy and Schulz, *Postgrad. Med.*, 1956, **19**, 403.
24. Shapiro, *J. Amer. med. Ass.*, 1956, **160**, 30.
25. Ambrus, Ambrus, Bauer and Noell, *J. Pharmacol.*, 1957, **119**, 129.
26. Gottschalk, Kapp, Ross, Kaplan, Silver, Macleod, Kahn, Van Maanen and Acheson, *J. Amer. med Ass.*, 1956, **161**, 1054.
27. Hill, *Brit. med. Bull.*, 1951, **7**, 278.
28. Hoch, from *Drugs of Choice*, C. V. Mosby Company, St. Louis, 1958.
29. Mushin and Mapleson, *Brit. J. Anaesthesia*, 1957, **29**, 249.
30. Raymond, Lucas, Beesley and O'Connell, *Brit. med. J.*, 1957, **2**, 63.
31. Lasagna and Imboden, *Fed. Proc.*, 1956, **15**, 451.
32. Lasagna, Von Felsinger and Beecher, *J. Amer. med. Ass.*, 1955, **157**, 1006.
33. Laties and Weiss, *J. chron. Dis.*, 1958, **7**, 500.
34. Loomis and West, *J. Pharmacol.*, 1958, **122**, 525.
35. Von Felsinger, Lasagna and Beecher, *J. Amer. med. Ass.*, 1955, **157**, 1113.
36. Gay and Carliner, *Bull. John Hopkins Hosp.*, 1949, **84**, 470.
37. Glaser and Hervey, *Lancet*, 1951, **2**, 749.
38. Report of Study by Army, Navy, Air Force Motion Sickness Team. *J. Amer. med. Ass.*, 1956, **160**, 755.
39. Lasagna and Von Felsinger, *Science*, 1954, **120**, 359.
40. Clark, *Edinb. med. J.*, New Series, 1935, **42**, 1.
41. Gaddum, *Proc. R. Soc. Med.*, 1954, **47**, 195.
42. Beecher, *J. Amer. med. Ass.*, 1955, **158**, 399.
43. Beecher, *Amer. J. Physiol.*, 1956, **187**, 163.
44. Beecher, *J. Amer. med. Ass.*, 1955, **159**, 1602.
45. Beecher, Keats, Mosteller and Lasagna, *J. Pharmacol.*, 1953, **109**, 393.
46. Fischer and Dlin, *Amer. J. med. Sci.*, 1956, **232**, 504.
47. Friend, O'Hare and Levine, *Amer. Heart. J.*, 1954, **48**, 775.
48. Houston, *Ann. int. Med.*, 1938, **11**, 1416.
49. Lasagna, Laties and Dohan, *J. clin. Invest.*, 1958, **37**, 533.
50. Moyer, *Arch. intern. Med.*, 1956, **96**, 608.
51. Wolf, *J. clin. Invest.*, 1950, **29**, 100.
52. Wolf and Pinsky, *J. Amer. med. Ass.*, 1954, **155**, 339.
53. Batterman and Grossman, *ibid.*, 1955, **159**, 1619.
54. Cornell Conference on Therapy, *Amer. J. Med.*, 1954, **17**, 722.
55. Cornell Conference on Therapy, *ibid.*, 1947, **2**, 296.
56. Cornell Conference on Therapy, *N.Y. St. J. Med.*, 1946, **46**, 1.
57. Gold, *Amer. J. Med.*, 1952, **12**, 619.

58. Corcoran, Dustan and Page, *Ann. int. Med.*, 1955, **43**, 1161.
59. Aravanis and Luisada, *Ann. int. Med.*, 1956, **44**, 1111.
60. Bello and Turner, *Amer. J. med. Sci.*, 1956, **232**, 194.
61. Bepler and Rogers, *ibid.*, 1957, **234**, 459.
62. Hailman, *J. Amer. med. Ass.*, 1953, **151**, 1430.
63. Koteen, *Ann. int. Med.*, 1957, **47**, 978.
64. Abstract, *Antibiotic med. and Clin. Therapy*, 1958, **5**, 615.
65. Wolff, *Ann. int. Med.*, 1947, **27**, 944.
66. Wolff, *Proc. Assoc. Res. nerv. and ment. Dis.*, 1950, **29**, 1059.
67. Greiner, Gold, Cattell, Travell, Bakst, Rinzler, Benjamin, Warshaw, Bobb, Kwit, Modell, Rothendler, Messeloff and Kramer, *Amer. J. Med.*, 1950, **9**, 143.
68. Chernish, Gruber and Kohlstaedt, *Proc. Soc. exp. Biol., N.Y.*, 1956, **93**, 162.
69. Armitage, *Amer. J. Public Health*, 1958, **48**, 1395.
70. Bross, *J. chron. Dis.*, 1958, **8**, 349.
71. Bross, *Ann. int. Med.*, 1955, **43**, 442.
72. Fisher, from *Design of Experiments*, Oliver and Boyd, Ltd., London, 1949.
73. Hume, *Lancet*, 1957, **2**, 1049.
74. Jellinek, *Biometrics*, 1946, **2**, 87.
75. Luykx, *J. Amer. med. Ass.*, 1949, **141**, 195.
76. Mainland, *Ann. rheum. Dis.*, 1955, **14**, 337.
77. Mainland, *Amer. Heart. J.*, 1958, **55**, 644.
78. Marshall and Merrell, *Bull. John Hopkins Hosp.*, 1949, **85**, 221.
79. Symposium, *Biometrics*, 1952, **8**, 206.
Lasagna, *J. chron. Dis.*, 1955, **1**, 353.
Lasagna, Mosteller, Von Felsinger and Beecher, *Amer. J. Med.*, 1954, **16**, 770.
Page and Corcoran, *Circulation*, 1956, **14**, 868.
Pepper, *Amer. J. med. Sci.*, 1943, **206**, 703.
Woolmer, *Proc. R. Soc. Med.*, 1959, **52**, 98.

RESEARCH PAPERS

PAPER CHROMATOGRAPHY OF SOME TISSUE AMINES

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Received April 14, 1959

When solutions of adrenaline, noradrenaline, histamine, 5-hydroxytryptamine and related amines in trichloroacetic, trifluoroacetic or picric acid are chromatographed in various organic solvent systems, the active material divides into two areas. If a basic amino acid is included in the solution before chromatography, the separation becomes more complete. It is suggested that the second area consists of a loose complex between the amine and the corresponding acid. When solutions of the amines in hydrochloric, acetic or oxalic acid are chromatographed, the active material resides only in one area.

When extracts of animal tissues are tested for some of their biologically active constituents, usually they are freed from blood and other protein, often by treatment with trichloroacetic acid. Occasionally such extracts are also subjected to paper chromatography. When the chromatograms are developed the active areas are sometimes found to be split into two. For example, the two spot-formation was reported in 1952 for adrenaline when extracts of adrenal glands were made in this way and chromatography was carried out in various organic solvents[1]. In a similar manner, histamine was found in 1954 to reside in two distinct areas when extracts of mast cell tumours were chromatographed[2]. The present work is a further study of this phenomenon and includes the testing of extracts of tissues rich in 5-hydroxytryptamine (5-HT) or its derivatives.

Methods

Solutions (0·1 per cent, w/v) of pure synthetic amines possessing adrenaline-like histamine-like or 5-HT-like properties were made either in 0·33 N hydrochloric acid, or in 5 per cent trichloroacetic, trifluoroacetic, picric, acetic or oxalic acids. In some experiments, arginine, lysine or ornithine (1 per cent w/v) were also included in the solutions. Ten μg. of each amine were applied to paper (Whatman No. 1) and ascending chromatograms run for 18 hours. The solvents used were *n*-butanol: acetic acid: water (4 : 1 : 5), *iso*propanol: ammonia: water (20 : 1 : 2), *n*-butanol saturated with N HCl, and *n*-propanol: water (1 : 1·5).

The spray developers included aqueous potassium iodate[3], alkaline potassium ferricyanide and formaldehyde[4], *p*-nitroaniline diazo reagent[5], and Folin and Ciocalteu reagent to detect adrenaline-like substances, Pauly diazo-reagent, chiefly for detecting histamine, and Ehrlich's and N.N.C.D. reagents, to detect 5-HT and its derivatives.

For testing the biological activity of certain locations on the paper, chromatograms were made in duplicate, one paper being developed by the spray reagent whilst the other was used for elution of the active areas

with 0·01N HCl. The eluates were then tested on the blood pressure of a spinal cat for adrenaline-like activity, on the isolated ileum of the guinea pig for histamine, or on the isolated atropinised uterus of a rat in oestrus for 5-HT-like activity. In some experiments, extracts of tissues rich in adrenaline, noradrenaline, histamine, 5-HT or tryptamine were similarly used.

Results

Adrenaline and Related Compounds

A solution of adrenaline in hydrochloric acid showed only one adrenaline spot at $R_F = 0{\cdot}32$ when chromatographed in the butanol : acetic acid : water solvent, whereas a similar solution in 5 per cent trichloroacetic acid showed two active areas at $R_F = 0{\cdot}32$ and 0·63. When each area from the latter chromatogram was eluted and assayed for adrenaline, the activity was almost equally divided between the two areas. If the excess trichloroacetic acid was removed from the solution by ether extraction before chromatography, the intensity of the faster-running spot ($R_F = 0{\cdot}63$) was greatly reduced. That the effect was entirely due to the presence of trichloroacetic acid was shown by passing the solution through a column of the anion exchange resin, Amberlite IRA-400, chloride form. The filtrate containing adrenaline in hydrochloric acid, approximately 0·33N, gave only one spot on chromatography at $R_F = 0{\cdot}32$. Little or no loss in activity had occurred on passage of the solution through the resin. Since weakly acid phenols may form rather unstable compounds with trichloroacetic acid[6], it has been suggested that the additional faster-running spot is caused by such a complex formation between adrenaline and trichloroacetic acid[1]. The material in the faster-running area readily split up for simple elution of this area followed by rechromatography in the same solvent resulted in a single adrenaline spot at $R_F = 0{\cdot}32$. A trichloroacetic acid spot was then also detected at $R_F = 0{\cdot}90$ with a potassium iodide-iodate-starch developer.

The double-spot phenomenon with adrenaline also occurred when the other solvent systems were used. Solutions of adrenaline in trifluoroacetic or picric acids likewise yielded double spots of adrenaline, but those in oxalic, acetic or hydrochloric acid did not. When a basic amino acid such as lysine, arginine or ornithine was also present in the solution of adrenaline in trichloroacetic, trifluoroacetic or picric acid, more than 80 per cent of the adrenaline activity passed to the faster-running area ($R_F = 0{\cdot}63$).

The double-spot formation was similarly observed with solutions of the following adrenaline-like substances. (*a*) Dihydroxyphenylethanolamines and their α-substituted derivatives, such as noradrenaline, *N*-ethylnoradrenaline, *N-iso*propylnoradrenaline (isoprenaline), corbasil and α-ethylnoradrenaline, (*b*) dihydroxyphenylethylamines, such as dopamine and epinine, (*c*) monohydroxyphenylalkylamines such as tyramine, paredrine, *p*-sympatol and *m*-sympatol, (*d*) phenyl*iso*propanolamines such as propadrine and ephedrine, (*e*) the phenyl*iso*propylamine, amphetamine,

(*f*) the ketone, adrenalone, and (*g*) the amino acids, 3,4-dihydroxyphenylserine and 3,4-dihydroxyphenylalanine. The formation of double spots of noradrenaline and of each of the amino acids was achieved only when the amount of substance applied to the paper was increased at least 10 times, that is, to more than 100 μg. In all cases, the presence of a basic amino acid facilitated the separation of the active area into two and increased the amount found in the faster-running area.

When an extract of cat suprarenal glands in trichloroacetic acid was similarly chromatographed, the adrenaline was found to reside in two distinct locations ($R_F = 0{\cdot}32$ and $0{\cdot}64$). Likewise, an extract of a canine phaeochromocytoma showed on chromatography two distinct areas for noradrenaline ($R_F = 0{\cdot}25$ and $0{\cdot}62$). In this experiment, relatively large concentrations of both arginine and lysine were also detected on the chromatograms; these two basic amino acids no doubt aided the separation of the noradrenaline into two areas under the conditions used.

Histamine

When solutions of histamine in hydrochloric, acetic or oxalic acid were applied to paper and chromatograms were run in the butanol : acetic acid : water solvent, a compact spot of histamine was formed at $R_F = 0{\cdot}11$. However, solutions in trichloroacetic, trifluoroacetic or picric acid treated similarly showed two concentrated areas of histamine at $R_F = 0{\cdot}11$ and $0{\cdot}65$, with some trailing between the two locations. About 10 per cent of the biological activity resided in the faster-running area. As with adrenaline, the presence of a basic amino acid facilitated the formation and increased the intensity of the area at $R_F = 0{\cdot}65$ so that 60 per cent of the activity resided there, whereas partial removal of the trichloroacetic acid by ether extraction reduced its intensity. When an eluate of the faster-running area was re-run in the same solvent, the histamine now remained entirely in its lower location ($R_F = 0{\cdot}11$). Similar results were obtained with the other organic solvents used.

Extracts of tissues exceptionally rich in histamine, such as ox pleura, ox liver capsule, and mast cell tumours from dogs, behaved similarly to solutions of pure histamine and showed two areas of histamine on chromatography. In these experiments, relatively large concentrations of arginine were also detected on the chromatograms and no doubt aided the separation of the histamine into two areas. Recently, the two-spot formation of histamine has been noted when extracts of liver of a case of urticaria pigmentosa were subjected to chromatography[7].

Compound 48/80, a potent histamine-liberator *in vivo*, was next included in the mixture of histamine and trichloroacetic acid which was applied to the paper. This was done when it was found that the only amino acids to facilitate the formation of the faster-running area of histamine were the basic ones which had already been shown to be the only amino acids capable of releasing histamine from tissues[8]. In fact, compound 48/80 (20 μg.) completely separated the histamine (10 μg.) into the two areas, with more than 75 per cent of the histamine activity in the faster-running location. But tubocurarine, another histamine-liberator *in vivo*,

in amounts up to 2 mg. for every 10 μg. of histamine, failed to increase the histamine activity of the faster-running area and even did not remove the trail between the two histamine locations. Nevertheless, when both compound 48/80 or tubocurarine and a basic amino acid were contained in the mixture before chromatography, complete separation of the histamine occurred and over 80 per cent of the activity passed to $R_F = 0{\cdot}65$. The amounts of compound 48/80 or tubocurarine needed for this effect were as little as 3 μg. Two other histamine-liberators, ammonia and sodium hydroxide (0·1N), also effectively separated the histamine activity into two locations when added to solutions of histamine in trichloroacetic acid before chromatography.

5-Hydroxytryptamine

Solutions of 5-HT in trichloroacetic, trifluoroacetic or picric acid gave the two-spot formation when chromatographed in the solvents listed. In the butanol:acetic acid:water solvent system, for example, the 5-HT positions were at $R_F = 0{\cdot}38$ and 0·64, with about 30 per cent of the total activity residing in the faster-running area. When a basic amino acid was included in the mixture before chromatography, more than 70 per cent of the 5-HT activity passed to the area of $R_F = 0{\cdot}64$. When this area was eluted and the eluate re-run in the same solvent, a single spot characteristic of free 5-HT ($R_F = 0{\cdot}38$) was produced. Solution of 5-HT in acetic, oxalic or hydrochloric acid gave only one spot of 5-HT ($R_F = 0{\cdot}38$). When an extract of a carcinoid tumour (producing much 5-HT) was made in trichloroacetic acid and a chromatogram prepared as for the pure 5-HT solutions, there were two distinct locations of 5-HT ($R_F = 0{\cdot}36$ and 0·60), the fast-running area containing over 60 per cent of the 5-HT activity. Extracts made in acetone showed only one spot ($R_F = 0{\cdot}38$) when chromatographed similarly.

The formation of two spots of 5-HT also occurred in the other solvents used. Similarly, solutions of tryptamine or *NN*-dimethyl-5-HT (bufotenine) in trichloroacetic acid showed two distinct areas of each amine. Likewise, an extract of tomatoes[9] in trichloroacetic acid gave two tryptamine spots.

Discussion

The remarkable ability of trichloroacetic, trifluoroacetic or picric acid to extract biologically active amines from tissues may be related not only to their coagulative effect on protein but also to their participation in a loose complex which contains the amine and generally one or more basic amino acids. Detailed analyses of the tissue extracts used in the present experiments show that relatively large concentrations of either arginine or lysine or both are present, and these most probably play a part in deciding whether the active material resides in one or two areas on the chromatograms. Another factor is the concentration of the acid used, for its partial removal causes most of the active amine to be found in its normal location on the chromatogram. But, it can be shown that the two-spot formation does not occur when aqueous solvents (such as

8 per cent w/v sodium chloride) are used for chromatography. These observations therefore emphasise the need for great care in the preparation and interpretation of chromatograms of biologically active amines extracted from tissues.

In any one solvent system used in the present experiments, the R_F values of the faster-running spots of adrenaline, noradrenaline, histamine or 5-HT are similar (0·60 to 0·66). This suggests that for each of these amines a similar unstable complex is formed. Since these complexes on elution are physiologically active, they may have some biological importance.

References

1. Shepherd and West, *Nature, Lond.*, 1952, **169**, 797.
2. Riley and West, *ibid.*, 1954, **174**, 882.
3. Shepherd and West, *Brit. J. Pharmacol.*, 1951, **6**, 665.
4. Shepherd and West, *Nature, Lond.*, 1953, **171**, 1160.
5. Wickstrom and Salvesen, *J. Pharm. Pharmacol.*, 1952, **4**, 631.
6. Kendall, *J. Amer. chem. Soc.*, 1916, **38**, 1309.
7. Gardner and Tice, *Pediatrics*, 1958, **21**, 805.
8. Eldridge and Paton, *J. Physiol.*, 1954, **124**, 27*P*.
9. West, *J. Pharm. Pharmacol.*, 1958, **10**, 589.

EFFECT OF LOCAL ANAESTHETICS ON BARBITURATE SLEEPING TIME

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Received April 15, 1959

The effect of certain local anaesthetics on barbiturate sleep in the mouse and the guinea pig is described. One of a series of new local anaesthetics 1-(1-methyl-2-phenylcarbamoylethyl)pyrrolidine (WS 10) prolongs barbiturate sleep in the guinea pig 2·3 times at the optimal dose without obvious side effects; other closely related members of the same series diminish barbiturate sleep.

A wide variety of substances affect the actions of barbiturates, modifying both their dormitive and their anti-epileptic properties[1,2]. Various mechanisms have been suggested in explanation of these actions. For example, Lamson[3,4] demonstrated that certain intermediates of glucose metabolism potentiate the anaesthetic action of barbiturates such as hexobarbitone, barbitone, quinalbarbitone and thiopentone. Frommel[5–7] gave evidence that meprobamate potentiates the sedative action of phenobarbitone and other barbiturates. The intermediates of glucose metabolism, however, show no effect on the anaesthesia produced by ethanol or ether.

The potentiating action of the intermediates of glucose metabolism in the brain on barbiturates can be blocked by acetylcholine[8–11].

The present communication concerns the effect of certain local anaesthetics on the anaesthetic action of barbiturates, particularly phenobarbitone and pentobarbitone.

Experimental Methods

Guinea pigs, 400 to 1000 g., and preferably 500 to 600 g., were used. Animals under about 400 g., or over 1000 g. tended to give erratic results. Constant room temperature and relative humidity were found to be desirable for reproducible results. Temperatures of 21–24·5° and a relative humidity of 60–75 per cent were found to be optimal.

The same animals were re-used for sleep experiments after a minimum rest period of 5 days. Generally the intraperitoneal route was used, the barbiturate being injected first as a 1 per cent solution of the sodium salt in water, followed immediately by a 1 per cent solution of the hydrochloride of the local anaesthetic in water. In some experiments the local anaesthetic and the barbiturate was given by mouth. Essentially the same result was obtained irrespective of the route of administration.

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Each animal was placed in a separate cardboard box and the times noted at which the animal no longer resisted the dorsal position and then the time at which it reverted spontaneously to the ventral position.

The dose of barbiturate was kept constant, and that of local anaesthetic varied. The local anaesthetics used were the following: procaine, lignocaine, piperocaine, cinchocaine, WS 2, 4, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 51, 53, 54, 56, 88 and 295, the formulae of which are given in Table V.

Results

In the absence of barbiturates WS 10, 1-(1-methyl-2-phenylcarbamoylethyl)pyrrolicine, showed a regular and lignocaine an irregular dormitive effect in the guinea pig at doses between 30–50 mg./kg. (i.p.), see Table I. These two substances together with WS 23 and 295 were among the most potent of those which prolonged barbiturate sleeping times.

TABLE I

SLEEP INDUCED BY LOCAL ANAESTHETICS ALONE IN THE GUINEA PIG

	Dose of local anaesthetic i.p. in mg./kg.	Number	Sleeping time in minutes mean
WS 10	15	6	0
	30	6	0
	45	6	32
	60	6	0
	90	6	0 (3 animals convulsed)
Procaine	30	6	0
	60	6	0
	90	6	0
Lignocaine	30	6	0
	45	6	5
	60	4	12
	90	3	0 (convulsions)

Table II shows the prolonging effect of WS 10 at various doses on the dormitive action of pentobarbitone compared with known local anaesthetics.

The prolonging effect of WS 10 is seen at low doses, whereas higher doses of lignocaine and procaine are needed. The barbiturate sleep-prolonging effect has no direct relation to the local anaesthetic action of these drugs, since lignocaine is, for example, a stronger local anaesthetic, weight for weight, than WS 10 while procaine is about the same. Piperocaine shows practically no sleep-prolonging effect of barbiturates, even at toxic doses. Similarly, cinchocaine showed poor sleep-prolonging action in spite of its high local anaesthetic activity.

For WS 10 a maximum potentiation of 2·3 times was observed at 75 mg./kg. (i.p.). At higher doses spasmogenic and excitatory effects became marked, resulting in progressively reduced sleeping time as the dose was increased. Finally, true sleep observations became unreliable owing to the typical convulsions produced by toxic doses of local anaesthetics. With cinchocaine the high toxicity masked true sleep at low doses, so that the results are given here with reserve.

TABLE II

PROLONGATION OF PENTOBARBITONE, 10 MG./KG. I.P., SLEEPING-TIME IN THE GUINEA PIG

Dose local anaesthetic i.p. mg./kg.	No. of animals	Mean $\bar{X}$ sleeping time min.	Prolongation per cent	Standard deviation min.
W.S. 10 2·5	15	85·0	10·4	12·0
" " 5·0	18	115·6	50·5	10·6
" " 10·0	23	117·4	52·0	16·8
" " 15·0	24	149·4	97·6	15·9
" " 30·0	18	153·4	104·7	14·2
" " 45·0	18	177·3	130·0	15·1
" " 60·0	18	207·9	170·0	17·5
" " 75·0	28	254·7	231·0	22·0
" " 90·0	21	228·0	194·2	20·1
Procaine 30·0	12	107·0	38·9	9·8
" 60·0	22	143·1	60·5	21·4
" 90·0	22	121·6	41·7	27·0
" 120·0	12	105·0	36·4	15·1
Piperocaine 10·0	10	81·3	5·5	8·2
" 20·0	6	81·5	5·9	13·9
" 40·0	6	89·5	16·2	14·6
Cinchocaine 5·0	18	79·2	2·9	9·8
" 10·0	18	124·0	60·9	17·9
" 20·0	18	127·8	65·8	14·9
" 40·0	12	112·5	46·0	23·4
Lignocaine 15·0	12	113·3	47·1	16·1
" 30·0	18	139·6	82·1	11·0
" 45·0	18	157·3	105·3	15·5
" 60·0	18	164·7	113·8	15·5
" 75·0	15	142·8	86·7	14·3
" 90·0	15	142·3	87·4	17·0
Control Pento-barbitone 10·00	56	77·05	0	5·5

Parallel results with barbiturates other than pentobarbitone are reported in Tables III and IV. It was not thought of immediate interest to work with large enough numbers of animals to allow a statistical treatment of the data. The results obtained with phenobarbitone and WS 10 both by mouth are given in Table III.

TABLE III

PROLONGATION OF PHENOBARBITONE SLEEPING-TIME 30 MG./KG. BY MOUTH IN THE GUINEA PIG

Dose WS 10 mg./kg.	No. of animals	Sleeping time in minutes mean	Prolongation per cent
10	4	80	57
35	8	105	105
0	4	51	0
50	4	290	222
0	4	90	0
75	4	174	123
0	4	78	0
100	4	90	76
0	4	51	0

Since the number of animals was small, each experiment was made with a simultaneous control. In some of the preliminary siting experiments the room temperature and the relative humidity were not controlled,

so that the control values for sleep with each potentiating agent vary. But the results show that the general pattern is similar to that seen with pentobarbitone under more rigidly controlled conditions. A maximum prolongation is reached at about 50 mg./kg. of WS 10. Here again, on increasing the dose, the spasmogenic effect of the local anaesthetic appears, thus reducing the sleeping time. Table IV shows similar effects with hexobarbitone and WS 10 in the mouse.

TABLE IV

PROLONGATION OF HEXOBARBITONE SLEEPING-TIME 75 MG./KG. I.P. IN THE MOUSE

Dose WS 10 in mg./kg.	No. of animals	Sleeping time in minutes mean	Prolongation per cent
10 s.c.	5	100	29
0	5	77	0
20 s.c.	5	149	109
0	5	71	0
100 by mouth	5	104	39
0	5	75	0

To test the generality of this potentiating effect preliminary siting tests were made with some selected members of the same chemical series as WS 10 with the results given in Table V.

The results bring out some interesting relations between structure and activity.

DISCUSSION OF STRUCTURE: ACTIVITY RELATIONS

Barbiturate sleeping-time prolongation appears often in this type of local anaesthetic but does not seem to be a direct function of the local anaesthetic activity of these substances[12,13]. WS 15 and WS 17 show about the same local anaesthetic activity but possess widely differing properties of barbiturate sleeping-time prolongation. Further, WS 13, 15 and 25 are all more powerful local anaesthetics than WS 10, yet are weaker in prolonging sleeping-time. Substitution in the benzene ring seems, in general, to increase the local anaesthetic activity but sometimes to lower the barbiturate effect.

In general, the presence of a carbamoyl group seems to favour sleep-prolongation, as shown by the high activity of WS 10, 23, 295 and lignocaine, and by the lower activity of the ester type of local anaesthetic like procaine and piperocaine. This must be qualified by remarking that the presence of a morpholino group in the side chain, as in WS 51, 53, 54, 56 seems to neutralise this activity of the carbamoyl group without at the same time lowering significantly the local anaesthetic activity.

It is commonly assumed for structure: activity relation purposes that, in local anaesthetics of the above type, the pyrrolidin-1-yl and piperidino groups are interchangeable without appreciably altering the pharmacology.

A comparison between the sleep-prolonging effect shown by WS 2 and 4, for example, demonstrates this generalisation may be unsound, since WS

TABLE V

30 MG./KG. PHENOBARBITONE BY MOUTH IN THE GUINEA PIG

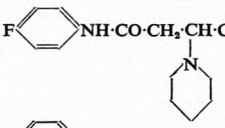
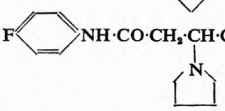
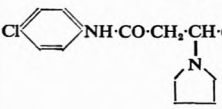
Number of anaesthetic	Formula	Number of guinea pigs	Dose of anaesthetic orally mg./kg.	Sleep prolongation per cent
WS 2		4 4 4	10 35 100	36 160 – 20
WS 4		4 4 4	10 35 100	– 39 – 5 – 20
WS 13 ..		4 4 4	10 35 100	53 35 – 60
WS 24 ..	*p*–i–$C_3H_7OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 40 – 33 – 100
WS 25 ..	*p*–i–$C_3H_7OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO_2{\cdot}CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 31 20 47
WS 22 ..	*p*–n–$C_4H_9OOC{\cdot}C_6H_4{\cdot}NH.CO{\cdot}CH_2.CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 32 – 19 – 100
WS 23 ..	*p*–n–$C_4H_9OOC{\cdot}C_6H_4{\cdot}NH.CO{\cdot}CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 75	79 124 40
WS 56 ..	*p*–n–$C_4H_9OOC{\cdot}C_6{\cdot}H_4{\cdot}NH{\cdot}CO{\cdot}CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 33 – 36 – 100
WS 15 ..	*p*–$C_2H_5OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO.CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 100	144 20 – 59
WS 17 ..	*p*–$C_2H_5OOC{\cdot}C_6H_4.NH{\cdot}CO.CH_2.CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 67 – 64 – 100
WS 54 ..	*p*–$C_2H_5OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}CH_2{\cdot}CH{\cdot}CH_3$ 	4 4 4	10 35 100	– 79 – 47 – 41

TABLE V—*continued*

Number of anaesthetic	Formula	Number of guinea pigs	Dose of anaesthetic orally mg./kg.	Sleep prolongation per cent
WS 19 ..	*p*-$CH_3OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}CH_2{\cdot}CH(N\langle CH_2CH_2CH_2CH_2\rangle){\cdot}CH_3$	4 4 4	10 35 100	− 84 − 87 − 84
WS 20 ..	*p*-$CH_3OOC{\cdot}C_6H_4{\cdot}NH{\cdot}CO{\cdot}CH_2{\cdot}CH(N\langle CH_2CH_2CH_2CH_2\rangle){\cdot}CH_3$	4 4 4	10 35 100	41 95 − 99
WS 53 ..	*p*-$CH_3OOC{\cdot}C_6H_4{\cdot}NH.CO{\cdot}CH_2{\cdot}CH(N\langle CH_2CH_2OCH_2CH_2\rangle){\cdot}CH_3$	4 4 4	10 35 100	− 13 − 27 − 48
10 MG./KG. I.P. PENTOBARBITONE IN THE GUINEA PIG				
WS 10 ..	$C_6H_5.NH.CO.CH_2.CH(N\langle CH_2CH_2CH_2CH_2\rangle).CH_3$	23 18 28 21	10 45 75 90	52 130 231 194
WS 88 ..	$C_6H_5.NH.CO.CH_2.CH(N\langle CH_2CH_2CH_2CH_2CH_2\rangle).CH_3$	5 5 5 5	10 35 50 90	− 15 51 7 − 28
WS 295 ..	$C_6H_5.NH.CO.CH_2.CH(N(C_2H_5)_2).CH_3$	7 7 7 5	10 25 50 90	84 146 149 − 44
WS 51 ..	$C_6H_5.NH.CO.CH_2.CH(N\langle CH_2CH_2OCH_2CH_2\rangle).CH_3$	7 7 5	30 60 90	− 4 − 4 51

4 shows a sleep-diminishing effect, while WS 2 gives a positive action. Both substances are about equal in their local anaesthetic activity. Yet the only chemical difference between the two types of compound lies in the pyrrolidin-1-yl and piperidino groups.

WS 24 and 25 show the same type of effect but in reversed order, the compound containing the piperidino group (WS 24) showing, in this case no prolonging action, whereas the pyrrolidin-1-yl compound (WS 25) shows moderate activity. Similar effects are seen in the compounds WS 22 and 23, WS 15 and 17.

Thus, the pharmacological action of even such similar groups as the piperidino and pyrrolidin-1-yl groups situated in identical basic structures is still unpredictable, as judged by our methods. It is possible that the study of the pharmacological properties of the (+)- and (−)-isomers of

these substances might throw further light on these relationships which are shown in Table V.

These experiments were begun in the Institut de Thérapeutique Expérimentale, Ecole de Médecine, Geneva, Switzerland (Directeur, Professeur Edouard Frommel) and completed during a visiting professorship held by one of us (A.E.W.S.) during the academic year 1957–58 at the University of Illinois Professional Colleges, Chicago 12, U.S.A., to whom thanks are due for laboratory facilities and a research grant.

References

1. Richards and Taylor, *Anesthesiology*, 1956, **17**, 414.
2. Frommel, Bischler, Gold, Favre and Vallette, *Helv. Physiol. Acta*, 1947, **5**, 65.
3. Lamson, *Science*, 1949, **110**, 690.
4. Lamson, *Trans. Assoc. Amer. Physicians*, 1951, **64**, 237.
5. Frommel and Fleury, *Helv. Physiol. Pharmacol. Acta*, 1957, **15**, 426.
6. Frommel and Fleury, *Praxis*, 1957, **46**, 1129.
7. Frommel, Gold and Fleury, *J. Suisse Méd.*, 1957, **87**, 1480.
8. Greig and Holland, *Arch. Biochem.*, 1949, **23**, 370.
9. Holland and Greig, *Amer. J. Physiol.*, 1950, **162**, 1950.
10. Lindvig, Greig and Petersen, *Arch. Biochem.*, 1951, **30**, 241.
11. Holland and Greig, *ibid.*, 1952, **39**, 77.
12. Wilder Smith, *Helv. chim. Acta*, in press.
13. Wilder Smith, *ibid.*, in press.

THE CHEMISTRY OF THE *ARISTOLOCHIA* SPECIES. PART V. A COMPARATIVE STUDY OF ACIDIC AND BASIC CONSTITUENTS OF *A. RETICULATA* LINN., *A. SERPENTARIA* LINN., *A. LONGA* LINN. AND *A. INDICA* LINN.

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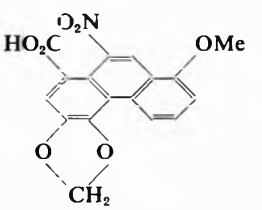
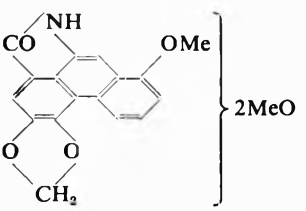
Received May 19, 1959

Aristolochic acid (I) was present in single samples of *A. longa* and *A. indica*, in two samples of *A. serpentaria*, and four samples of *A. reticulata*. Aristo-red (II) was present only in the samples of *A. reticulata* and *A. serpentaria*. Contrary to numerous reports of alkaloids in *Aristolochia* species, the current investigation has revealed the presence of only negligible amounts in the samples of *A. indica*, *A. longa*, *A. reticulata* and *A. serpentaria* examined. A flavone isolated from *A. reticulata* has been identified as isorhamnetin.

In Part III[1] acidic material from the roots and rhizomes of *A. reticulata* was shown to contain both aristolochic acid (I)[2,3] and aristo-red (II) whereas only the former was found in *A. indica*. Aristolochic acid and aristo-red have now also been separated by fractional crystallisation of



(I)



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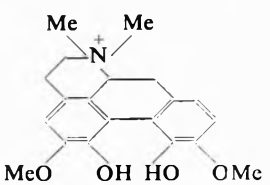


(III)

the acid isolated from *A. serpentaria*, and identified by comparison with authentic samples. The controversial reports of Pohl[4], who described the presence of aristolochin (≡ aristolochic acid[5]), and of Hesse[6], who was unable to identify aristinic acid (≡ aristolochic acid) in *A. longa*, have been resolved by the isolation of aristolochic acid (I) in good yield from this source. This, and other data summarised in Table I, indicates that aristolochic acid is characteristic of all *Aristolochias* so far examined. Aristo-red (II) is not present in *A. longa* and, like aristolactone[7], appears to be a characteristic constituent only of the North American *Aristolochias*.

TABLE I

ACIDS AND LACTONE CONSTITUENTS OF *Aristolochia* SPECIES

Source	Aristolochic acid (per cent)	Secondary acid constituents	Aristolactone (per cent)	Ref.
A. argentina, Griseb.	•	–	–	6
A. bracteata, Retz.	0·01	–	–	9
A. clematitis, Linn.	•	aristolochic acid–II	–	2; 3; 10
A. debilis, Sieb. et Zucc.	•	–	–	11
A. indica, Linn.	0·013	–	–	12
	0·070	0	0	1; 7
A. kaempferi, Willd.	•	–	–	13
A. longa, Linn.	0·20	0	0	7; present work
A. reticulata, Linn.	0·022	aristo-red	0·158	1; 7
A. serpentaria, Linn.	0·046	aristo-red	0·091	7; present work
	0·92	–	–	31
A. sipho, l'Hérit.	0·30	–	–	5
A. maxima, Jacq.	•	–	–	31
A. pandurata, Jacq.	•	–	–	31

* Present in unstated amount
– No specific search recorded
0 Substance absent

Cavallito and Bailey[8] isolated a crystalline substance, $C_{46}H_{11}O_7N$, from *Asarum canadense var. reflexum* (fam. Aristolochiaceae) which they termed substance B, the properties of which strongly suggest identity with aristolochic acid (see Table II).

TABLE II

PROPERTIES OF SUBSTANCE B AND ARISTOLOCHIC ACID

	Substance B	Aristolochic acid
Appearance	Yellow needles	Yellow needles
m.p.	Darkens between 230–260° without melting	Darkens around 240–250° then melts at 275° (decomp.)
Analyses	Found: C, 58·2 H, 3·5 N, 4·55 per cent	Requires C, 59·8 H, 3·2 N, 4·1 per cent
Ultra-violet absorption spectrum	λmax. (mμ) (based on $C_{17}H_{11}O_7N$) / ε	λmax (mμ) / ε
	250 / 29,325	223 / 30,000
	318 / 12,820	250 / 29,400
	390 / 6,615	318 / 13,100
		390 / 7,300

Alkaloids have been reported from time to time to be present in various *Aristolochia* species, since Feneulle[14] and Chevallier[15] described the isolation of a bitter yellow substance from *A. serpentaria*. Neither author claimed that this substance was basic, though later, Ferguson[16] indicated that it was probably identical with the bitter yellow crystalline base, aristolochine, which he obtained from *A. reticulata*. Winkler[17], on the other hand, claimed that the yellow crystalline acid from *A. clematitis*, which was undoubtedly aristolochic acid, was also identical with the bitter obtained by Feneulle and Chevallier. Hesse[6] reported the isolation from *A. argentina* and *A. indica* of an amorphous base, aristolochine, different from Ferguson's aristolochine, and also stated that alkaloids were not present in *A. longa*. Controversial reports are

also on record concerning the presence[18] or absence[19] of alkaloids in *A. cymbifera.*

A base was also reported by Dymok and Warden[20] in *A. indica* and later isolated in crystalline form, $C_{17}H_{19}O_3N$, from the same source by Krishnaswamy, Manjunath and Rao[12,21] in a yield of 0·05 per cent. The alkaloid which was also named aristolochine gave a crystalline hydrochloride, picrate (m.p. 222° decomp.) and picrolonate, showed the presence of one methoxyl group, an *N*-dimethyl group, and exhibited weakly acidic properties (soluble in sodium hydroxide, but insoluble in sodium carbonate), but gave no ferric chloride reaction. No detailed structural analysis has been reported. More recently, Tomita and Kura[13] have described the isolation of an aporphine type base, magnoflorine (III)[22] from *A. debilis* Sieb. et Zucc. and *A. kaempferi* Willd.

Plants of the genus *Aristolochia* are reported to have been held in high esteem on account, among other things, of their value in childbirth[23], and the common name Birthwort for certain species is obviously derived from these traditional uses. Some credence to the use of *Aristolochia* for this purpose is given by the work of Shaw[24], who reported that *A. elegans* contained an alkaloid which caused contraction of the uterus. We have accordingly examined a number of *Aristolochias* for alkaloids of potential value as oxytocics.

Single samples only of *A. longa* and *A. indica*, but two samples of *A. serpentaria* (contaminated with *Hydrastic canadensis*, from which it was separated as described below), and four samples of *A. reticulata*, received over a period of six years, were available to use. We are indebted to Dr. F. Fish and Mr. P. F. Nelson of this College for confirming the authenticity of the species designation of the samples. Index Kewensis lists four species of *Aristolochia longa*, and we wish to thank Dr. C. R. Metcalfe of the Royal Botanic Gardens, Kew, for his further confirmation of the authenticity of our sample as *A. longa* Linn.

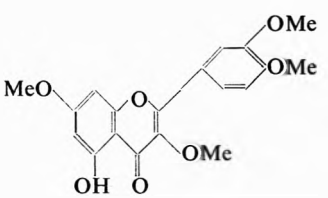
The ethanolic extract from *A. reticulata* gave only negligible amounts of a dark chloroform-soluble base, but precipitation of the residual aqueous liquor gave a crude reineckate, which was purified by chromatography from acetone on alumina. Decomposition of the pure reineckate[25], $C_{17}H_{20}O_3N[Cr(SCN)_4(NH_3)_2]\cdot 3H_2O$, yielded a hygroscopic partly crystalline base chloride, which gave a strongly positive reaction with Mayers reagent, but analysed only indifferently to the formula $C_{17}H_{20}O_3NCl$. A picrate, m.p. 178–179·5°, insufficient for analysis, was also obtained. Thus, although the molecular formula corresponds to that of the base reported in *A. indica*[12,21] inconsistency of the picrate melting points suggests that they are not identical.

A. indica yielded only a very small quantity (0·0007 per cent) of a yellow ether-soluble crystalline base, m.p. 339–342° (decomp.). The substance which was only weakly basic, fluoresced under ultra-violet light, and analysed as $C_{25}H_{23}O_{10}N$, again indicating non-identity with the alkaloid previously described[12,21]. Water-soluble alkaloids were absent. It is perhaps significant that the sample of root examined contained a higher percentage of aristolochic acid than reported by Krishnaswamy

and others[12] (see Table I), and since the acid ($C_{17}H_{11}O_7N$) would appear to be biogenetically related to the base they described ($C_{17}H_{19}O_3N$), our failure to find this alkaloid may possibly be explained by the time of year at which the plant material was collected. This aspect of the problem is being examined further.

Examination of the sample of *A. serpentaria* before extraction revealed that it was contaminated with *Hydrastis canadensis* root, and despite hand picking of this sample to remove contaminants, it yielded on extraction small quantities of hydrastine, together with a second base. Hydrastine was identified by analysis, melting point and ultra-violet absorption spectrum, but yielded a picrate, the melting point of which, 149°, was not in agreement with the reported values of 184°[26] and 190°[27]. Preparation of authentic hydrastine picrate from a sample of Liquid Extract of Hydrastis B.P.C. 1949 showed the melting point to be 149°. The second base, m.p. 178–179° was formulated by analysis as $C_{18}H_{15}O_{10}N$, and is not therefore identifiable with berberine or canadine the other known constituents of *Hydrastis canadensis*[28], or indeed any other known base. It was only weakly basic and showed an ultra-violet absorption maxima at 281·5 mμ (ϵ 12,030) and 353 mμ (ϵ 13,365) of the berberine type[29]. A second sample of *A. serpentaria*, which was similarly contaminated with *Hydrastis canadensis* yielded both hydrastine and berberine (in approximately equal amounts as usually found), but failed to yield the alkaloid $C_{18}H_{15}O_{10}N$. It is not clear, therefore, whether this base is present in *A. serpentaria* or derives from some further contaminant. In agreement with the findings of Hesse[6], no alkaloids were found in *A. longa*.

Further fractionation of the crude ethanol-soluble material from *A. reticulata*, isolated as described in Part III[1], yielded a small quantity of a yellow crystalline amphoteric substance, $C_{16}H_{12}O_7$, m.p. 318–322°, raised to 324° on repeated sublimation. The product was soluble in concentrated sulphuric acid to give a deep yellow solution, gave a greenish-brown colour with ferric chloride, and showed maxima in the ultra-violet at 255 mμ (ϵ 21,150), 307 mμ (ϵ 7,950) and 371 mμ (ϵ 22,100), all characteristic of a hydroxyflavone. Zeisel determination showed the



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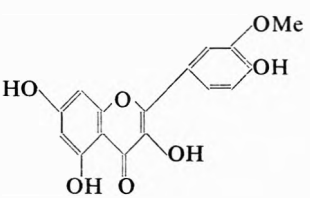

(V)

presence of one methoxyl group, and it was identified as a tetrahydroxy-methoxyflavone by conversion to the corresponding tetra-acetate, m.p. 214–215°, with acetic anhydride-pyridine. Diazomethane, on the other hand, provided evidence of a non-reactive hydroxyl, typical of 5-hydroxy-flavones[30] giving 5-hydroxy-3,3′,4′,7-tetramethoxyflavone (quercetin-3,3′,4′,7-tetra-methyl ether (IV)) as shown by melting point, ferric

chloride reaction, ultra-violet absorption spectrum and elementary analysis.

Identification of the parent monomethoxytetrahydroxyflavone, however, was not immediately possible, since although all five possible monomethoxyquercetins are known, the observed constants of our own product and its tetra-acetate did not conform with those of the derivatives for which corresponding data is available (Table III).

TABLE III

MONOMETHOXYQUERCETINS AND THEIR TETRA-ACETATES

Substance	m.p. (° C)	Ref.	λmax	log ε	Ref.	Tetra-acetate m.p. (° C)	Ref.
Quercetin-7-methyl ether (Rhamnetin) ..	294–296	39	256	4·40	32	186–188	39
	292–293	40	371	4·41	32	186–187	40
	>300	34				190–192	34
	290–294	42				183–185	42
Quercetin-3'methyl ether (Isorhamnetin) ..	296	41	255	—	36	198–199	41
	305	37	365–380	—	36	205–207	37
	307	38	(flat)			205	38
	295	35				198–200	35
Quercetin-4'-methyl ether	240	43				202	43
	259–260	30				203–204	30
Quercetin-5-methyl ether			254	4·30	32		
			369	4·25	32		
Quercetin-3-methyl ether	272–273	33	258	4·31	32		
			360	4·31	32		
Quercetin-*x*-methyl ether	318–322		255	4·32		214–215	
(present work)	(block)		370–372	4·34		(block)	

The considerable variation of recorded melting points is due to the fact that they are accompanied by decomposition, which makes them unreliable for characterisation purposes. Nevertheless, it would appear improbable that our own product is either the 3- or the 4'-methyl ether, whilst the 5-methyl ether is also excluded since diazomethane would yield pentamethoxy- and not the tetramethoxy-quercetin. The former conclusion is substantiated by the instability of the parent compound in ethanolic sodium ethoxide, which can be followed spectroscopically and is characteristic of flavones with unsubstituted hydroxyls in both the 3- and 4'-positions[32]. The ultra-violet spectrum in ethanolic sodium ethoxide (Fig. 1) differs from that recorded for rhamnetin (inflection at 238 mμ, log ε 4·35; λmax 294, log ε 4·11; λmax 358, log ε 3·98)[32]. There are no published spectra for isorhamnetin under the same conditions. The spectrum in ethanol is unaffected by boric acid-sodium acetate. Spectral shifts with the latter reagent are typical of vicinal dihydroxy compounds[46], and failure to elicit a shift is indicative of a methoxyl in the 3'-position and hence of isorhamnetin (V). Identity with isorhamnetin also seems to present the most reasonable conclusion on grounds of melting point (Table III) and ultra-violet absorption spectra, for although the spectra of rhamnetin and isorhamnetin are very similar, the former has a very broad minimum in the 300 mμ region[32], whereas the latter has a sharp minimum near 290 mμ[44].

An authentic sample of isorhamnetin could not be isolated by the reported method[45] from powdered red squill. Samples of synthetic isorhamnetin and its tetra-acetate were obtained, however, through the kindness of Professor G. Tappi[36]. In appearance, they were identical with our own products. Microblock melting points of 318–320° and 210–211° respectively were also in excellent agreement, whilst the ultra-violet absorption spectra of the synthetic isorhamnetin and our own flavone were superposable (Fig. 1), thus confirming identity.

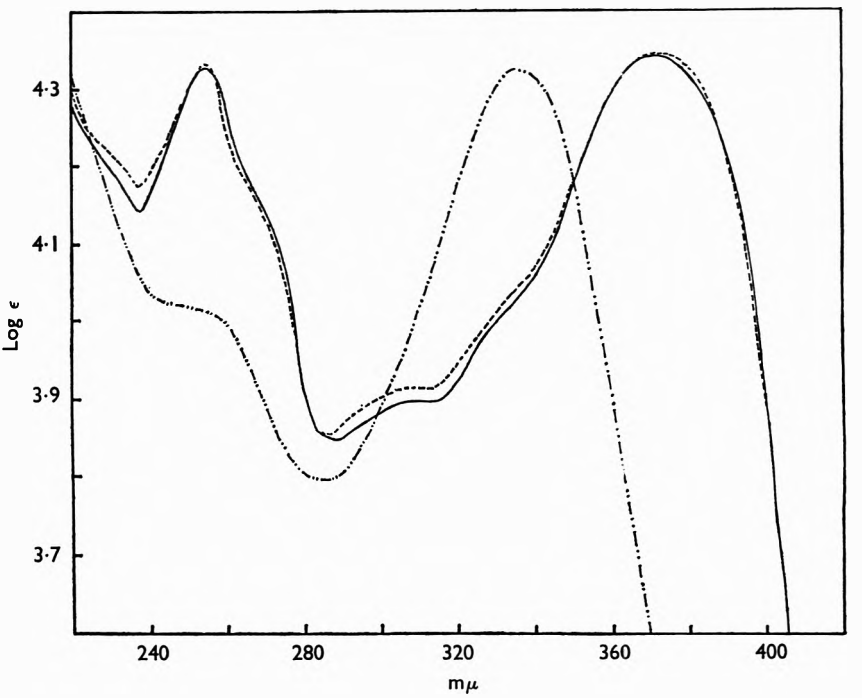

FIG. 1. Ultra-violet absorption spectra. Isorhamnetin ----. Flavone from *A. reticulata* ——. Flavone from *A. reticulata* in ethanolic sodium ethoxide – ··–.

EXPERIMENTAL

Melting points are uncorrected. Ultra-violet absorption spectra were determined in absolute ethanol on a Hilger Uvispek photoelectric spectrophotometer. R_F values were determined on Whatman No. 1 paper with 4:1 ethanol:5 per cent formic acid as solvent. We thank Mr. W. McCorkindale and Dr. A. C. Syme for microanalyses.

Extraction of A. longa

The dried root (3·01 kg.; No. 60 powder), previously defatted with light petroleum (b.p. 40–60°), was extracted with ethanol by cold percolation to give a dark orange extract (10 l.). During concentration, the yellow crystalline acid which separated out (7·12 g.) was repeatedly

filtered off before an almost black thick oil was obtained. The residue was acidified with dilute hydrochloric acid and the crude acids extracted with ether (treatment of the acid extract is reported below). Extraction of the ethereal solution with 2 per cent aqueous potassium hydrogen carbonate followed by acidification of the aqueous layer with dilute hydrochloric acid gave the crude acids. Fractional crystallisation of the bulked acid portions from glacial acetic acid gave eight fractions as yellow microcrystals (total weight 6·01 g.), each with m.p. 282–285° (decomp., block), R_F 0·90–0·94, λmax 250 (ε 30,600), 317 (ε 11,500), 390 mμ (ε 5,700), identical with aristolochic acid. Reduction with zinc and glacial acetic acid gave 9-amino-1-methoxy-5,6-methylenedioxy-8-phenanthroic lactam, m.p. 317° (block).

Treatment of acid extract. The solution was basified (dilute sodium hydroxide) and extracted with ether which on evaporation gave only a trace of a brown non-alkaloidal oil. The aqueous layer was acidified to congo red (dilute sulphuric acid) and treated with a saturated aqueous solution of ammonium reineckate. The resultant crude precipitate (4·078 g.) was completely insoluble in dry acetone.

Extraction of A. serpentaria

(*a*) The first sample of dried root and rhizome (4·34 kg.), from which appreciable quantities of *Hydrastis canadensis* root and rhizome and other adulterants had been removed, was reduced to a No. 60 powder, defatted with light petroleum (b.p. 40–60°) and percolated in the cold with ethanol until the percolate was pale brown (7 days). The thick black oil obtained on concentration was left at 0° for 4 days during which time β-sitosteryl-β-D-glucoside (1·88 g.) separated as a brown crystalline solid, m.p. 295–296° (after repeated recrystallisation from ethanol); acetate m.p. 166°. [Kind and Celentano[47] give m.p. of 295–297°, 157·5–168·5° respectively for β-sitosteryl-β-D-glucoside and its tetra-acetate.] The oily filtrate was dissolved in ether and the solution extracted with dilute hydrochloric acid (treatment of this acid extract is reported below). The crude acid fraction (3·64 g.), obtained from the ether solution by the method used for *A. longa*, was recrystallised from glacial acetic acid and gave aristolochic acid (2·00 g.), m.p. 283° (decomp.; block), R_F 0·915, identified further by conversion to 1-methoxy-5,6-methylenedioxy-9-nitrophenanthrene, orange needles, m.p. 212° (block). Concentration of the glacial acetic acid mother liquors gave, on repeated fractional recrystallisations from ethanol, red needles of aristo-red (35 mg.), m.p. 286·5–287·5° (block), R_F 0·78 (fluorescent spot in ultra-violet light). The ultra-violet absorption spectrum agreed with that reported in Part III[1].

Treatment of acid extract. The acidic solution was basified (dilute ammonium hydroxide) and extracted with ether, which on evaporation gave a dark-red partially crystalline oil (500 mg.). The benzene-soluble portion was chromatographed on alumina (5 in. × 0·5 in.) from benzene to give two fractions. The benzene-insoluble portion was non-alkaloidal.

Fraction 1. This came through as a compact yellow band which on evaporation gave pale yellow prism crystals (74 mg.), m.p. 178–179°

(decomp.; tube) (from ether or benzene). λmax 281·5 (ε 12,030), 353 mμ (ε 13,365). (Found: C, 53·6; H, 3·75; N, 3·6. $C_{18}H_{15}O_{10}N$ requires: C, 53·4; H, 3·7; N, 3·5 per cent.

Fraction 2. Removal of benzene gave pale yellow prism crystals of hydrastine (62 mg.), m.p. 132° (tube) (from methanol). λmax 297 mμ [*E* (1 per cent, 1 cm.) 196]. (Found: C, 65·6; H, 5·6; N, 3·7. Calculated for $C_{21}H_{21}O_6N$: C, 65·8; H, 5·5; N, 3·7 per cent.) [El Ridi, Khalifa and Mamoon[48] gave λmax 297 mμ [*E* (1 per cent, 1 cm.) 200, m.p. 132°]. The picrate had m.p. 148–149° (tube) (from ethanol). (Found: C, 53·2; H, 4·25. Calculated for $C_{21}H_{21}O_6N{\cdot}C_6H_2(NO_2)_3OH$: C, 52·95; H, 3·95 per cent.

(*b*) The second sample of defatted root and rhizome (4·20 kg., No. 60 powder), on concentration of the ethanolic extract, gave a thick black oil from which aristolochic acid, aristo-red and the acid extract were obtained as before.

Treatment of acid extract. The solution was basified (dilute sodium hydroxide) and extracted into ether which was, in turn, shaken out with sulphuric acid (2·5 per cent). On standing, the aqueous layer deposited orange crystals of berberine sulphate (1·037 g.), m.p. 288–290° (decomp.; block) (from alcohol-ether), λmax 267 [*E* (1 per cent, 1 cm.) 648], 351 mμ [*E* (1 per cent, 1 cm.) 609] (in 88 per cent ethanol). El Ridi, Khalifa and Mamoon[48] gave λmax 270 [*E* (1 per cent, 1 cm.) 610], 350 mμ [*E* (1 per cent, 1 cm.) 600] for berberine hydrochloride. (Found: C, 55·0; H, 4·2; N, 3·3; S, 7·2. Calculated for $C_{20}H_{17}O_4N{\cdot}H_2SO_4$: C, 55·4; H, 4·4; N, 3·2; S, 7·4 per cent.) The ether layer gave yellow prism crystals on removal of the solvent and chromatography from benzene on alumina (6 in. × 0·5 in.) yielded only hydrastine (1·097 g.), m.p. 132° (tube) (from methanol), 145° (tube) (from aqueous methanol). Both melting points have been reported[27] for hydrastine. The picrate melted at 148–149°.

The acid extracts from both samples of *A. serpentaria*, which had been basified and extracted with ether to remove basic material, were re-acidified to congo red (dilute sulphuric acid). Addition of a saturated aqueous solution of ammonium reineckate gave an amorphous dark brown solid (5·133 g.), which was only slightly soluble in dry acetone. Chromatography from dry acetone on alumina (38 g., 6·5 in. × 0·75 in.) gave a negligible quantity of pure reineckate.

Hydrastine picrate. A sample of hydrastine (0·82 g.), m.p. 132°, was obtained from Liquid Extract of Hydrastis B.P.C. 1949 (50 ml.) using the official assay method. The picrate was prepared by dissolving the base (0·1 g.) in hot methanol (10 ml.) and adding a saturated solution of picric acid in ethanol (5 ml.). It had m.p. 149° (decomp., tube) (from ethanol).

Extraction of A. reticulata

Treatment of acid extract. This was obtained by the method reported in Part III[1]. The acidic solution after a few days was basified (dilute ammonium hydroxide) and extracted with ether. After extraction of the latter with dilute hydrochloric acid to remove bases (treatment of this

fraction is reported below) the ether was evaporated to give yellow microcrystals of isorhamnetin (0·54 g.), m.p. 318–322° (decomp.; block) (from dioxan), raised to 324° on repeated sublimation at 300°/0·5 mm. (Found: C, 60·8; H, 3·7; O, 35·9; OCH_3, 11·5. Calculated for $C_{15}H_9O_6 \cdot OCH_3$: C, 60·8; H, 3·8; O, 35·45; OCH_3, 9·8 per cent.) λmax 255 (ε 21,150), 307 (ε 7,950), 370–372 mμ (ε 22,100). The ultra-violet absorption spectrum in ethanolic sodium ethoxide was carried out by the method of Jurd and Horowitz[32] allowing 1 hr. for reaction, λmax 335 (ε 21,200), 250–252 mμ (ε 10,480 flat). The spectrum in presence of boric acid-sodium acetate was recorded using the method of Jurd[46]. [A sample of isorhamnetin obtained from G. Tappi[36] had m.p. 318–320° (decomp., block), λmax 255 (ε 21,250), 307 (ε 8,150), 370–372 mμ (ε 22,120.] The flavone was also obtained by leaving the original acid extract at room temperature for several days when a green oily precipitate separated. Sublimation at 300°/0·5 mm. gave isorhamnetin (35 mg.).

Treatment of solution containing basic material. Successive extractions with ether then chloroform gave only traces of a dark-brown oil which gave slight positive tests with alkaloidal reagents.

The original acid extract, which had been basified and extracted with ether, was re-acidified to congo red (dilute sulphuric acid) and to it was added in excess a saturated aqueous solution of ammonium reineckate. The dark-brown crude base reineckate (31·2 g.) was dissolved in dry acetone and filtered from a large quantity of non-alkaloidal material. The deep red acetone solution was chromatographed from dry acetone on alumina (20 in. × 1·3 in.) and the single red band evaporated (water-bath, < 50°) to give a pink crystalline reineckate, m.p. 200°, (decomp., tube, insert at 195°) (from aqueous acetone). (Found: C, 37·8; H, 4·8; N, 14·8; OCH_3, 4·1, 4·0. $C_{16}H_{17}O_2N(OCH_3)$ $[Cr(SCN)_4(NH_3)_2] \cdot 3H_2O$ requires C, 38·3; H, 4·9; N, 14·9; OCH_3, 4·7 per cent.)

Decomposition of base reineckate. The reineckate (0·79 g.) was dissolved in dry acetone (20 ml.) and excess solution of silver sulphate added (0·599 per cent w/v, 35·0 ml.) followed by an equivalent volume of a solution of barium chloride (1·062 per cent w/v $BaCl_2 \cdot 2H_2O$; 15·50 ml.) when precipitation of silver reineckate had ceased. The combined precipitates of silver reineckate and barium sulphate were filtered off and washed thoroughly with distilled water; the combined filtrate and washings were evaporated to dryness (water-pump). This gave a very hygroscopic partially crystalline solid of doubtful purity (0·216 g.) from which inorganic material could not be completely removed. After repeated solution in water, it had $[\alpha]_D^{18}$ + 50·83, λmax 228 [*E* (1 per cent, 1 cm.) 367], 286 mμ [*E* (1 per cent, 1 cm.) 122]. (Found: C, 61·0; H, 9·2; N, 5·6. The expected base chloride $C_{17}H_{20}O_3NCl$ would require: C, 63·4; H, 6·3; N, 4·4 per cent.)

Base picrate. The base (50 mg.) was dissolved in water (2 ml.) and to this solution was added an aqueous solution of picric acid (0·66 per cent w/v, 4 ml.). Recrystallisation of the bulky product from ethanol was accompanied by decomposition and gave crystals (4 mg.), m.p. 178–179·5° (decomp., block).

Quercetin—3,3′4′,7-*tetramethyl ether.* Isorhamnetin (40 mg.) was suspended in dry ether (12 ml.) and an excess of diazomethane in dry ether added but no reaction occurred until a drop of water was added as catalyst[51]. After 3 hr., the excess diazomethane and solvent was removed giving long pale-yellow needles (19 mg.), of quercetin-3,3′4′,7-tetramethyl ether, m.p. 159–160° (tube) (from ethanol). (Found: C, 63·5; H, 5·4. Calculated for $C_{19}H_{18}O_7$: C, 63·7; H, 5·1 per cent.) λmax 254 (log ε 4·33), 269 (log ε 4·26), 353 mμ (log ε 4,305). [Gomm and Nierenstein[49] gave m.p. 159–160°. Briggs and Locker[50] gave λmax 254 (log ε 4·37), 269 (log ε 4·29), 352 mμ (log ε 4·34).]

Isorhamnetin—3,4′,5,7-*tetra acetate.* Isorhamnetin (40 mg.) was refluxed for 30 min. with acetic anhydride (2 ml.) and pyridine (2 ml.). To the cooled mixture, water was added dropwise to give white needles (72 mg.), which fluoresced brilliant green in ultra-violet light and had m.p. 214–215° (block) (from ethanol), λmax 239 (ε 20,650), 310 mμ (ε 16,050). (Found: C, 60·2; H, 4·5; OCH_3, 6·65. Calculated for $C_{22}H_{18}O_{10}$: C, 59·7; H, 4·1; OCH_3, 7·0 per cent. [A sample of isorhamnetin-3,4′,5,7-tetra-acetate obtained from G. Tappi[36] had m.p. 210–211° (block), λmax 240 (ε 21,750), 310 mμ (ε 16,700).]

Extraction of A. indica

A concentrated percolate was obtained as reported in Part III[1]. After extracting with ether, the acidic aqueous solution was basified (dilute sodium hydroxide) and again extracted with ether. This, on concentration gave yellow crystals (20 mg.) which fluoresced bright yellow in ultra-violet light and had m.p. 339–342° (decomp., block). (Found: C, 60·95; H, 4·75; N, 2·8. $C_{25}H_{23}O_{10}N$ requires: C, 60·4; H, 4·6; N, 2·8 per cent).

The aqueous alkaline layer from above was re-acidified to congo red (dilute sulphuric acid). Addition of a saturated aqueous solution of ammonium reineckate produced only a little crude reineckate (210 mg.) which gave a negligible quantity of pure material when chromatographed with dry acetone on alumina.

Acknowledgements. We thank the British Drug Houses Ltd., London, and Smith, Kline and French Laboratories, Philadelphia, for gifts of raw materials.

References

1. Coutts, Stenlake and Williams, *J. chem. Soc.*, 1957, 4120.
2. Pailer, Belohlav and Simonitsch, *Monatsh.*, 1955, **86**, 676.
3. Pailer, Belohlav and Simonitsch, *ibid.*, 1956, **87**, 249.
4. Pohl, *Arch. exp. Path. Pharmak.*, 1892, **29**, 282.
5. Rosenmund and Reichstein, *Pharm. Acta Helvet.*, 1943, **18**, 243.
6. Hesse, *Arch. Pharm.*, 1895, **233**, 684.
7. Steele, Stenlake and Williams, *J. chem. Soc.*, in the press.
8. Cavallito and Bailey, *J. Amer. chem. Soc.*, 1946, **68**, 489.
9. Dutta and Sastry, *Indian J. Pharm.*, 1958, **20** (10), 302.
10. Pailer and Schleppnik, *Monatsh.*, 1957, **88**, 367.
11. Tseng and Ku, *Acta Chim. Sinica*, 1957, **23** (2), 157.
12. Krishnaswamy, Manjunath and Rao, *J. Ind. chem. Soc.*, 1935, **12**, 476.
13. Tomita and Kura, *J. pharm. Soc., Japan*, 1957, **77**, 812.

14. Feneulle, *J. de Chimie med.*, 1826, **2**, 431.
15. Chevallier, *Journal de Pharmacie*, 1820, **6**, 565.
16. Ferguson, *Amer. J. Pharm.*, 1887, **59**, 481.
17. Winkler, *Jahrb. F. Prakt. Pharmacie.*, 1849, **19** (Neue Folge 16), 71.
18. Peckolt, *Pharm. Rundschau*, New York, 1893, **11**, 181.
19. Butte, *Journal des nouveaux remèdes* 1889, 460 (quoted by Castille, *J. Pharm. Belg.*, 1922, **4**, 141).
20. Dymok and Warden, *Pharm. J.* and *Transactions*, 1891 (3), **22**, 245.
21. Krishnaswamy and Manjunath, *J. Ind. chem. Soc.*, 1937, **14**, 39.
22. Tatsuhiko Nakano, *Pharm. Bull.* (*Japan*), 1954, **2**, 326, 329; Tomita and Kikuchi, *J. pharm. Soc., Japan*, 1956, **76**, 597; Tomita and Kugo, *ibid.*, 1956, **76**, 599.
23. Dawson, *Pharm. J.*, 1927, 396, 427.
24. Shaw, *Aust. J. Pharm.*, 1947, **28**, 857.
25. Dutcher, *J. Amer. chem. Soc.*, 1946, **68**, 419.
26. Henry, *The Plant Alkaloids*, 163, J. and A. Churchill Ltd., 1949.
27. Manske and Holmes, *The Alkaloids*, *IV*, Academic Press Inc., New York, 1954, p. 186.
28. Power, *Pharm. J.*, 1884–85 [iii], **15**, 297; 1885–86 [iii], **16**, 1092.
29. Skinner, *J. chem. Soc.*, 1950, 823.
30. Gupta and Seshadri, *J. chem. Soc.*, 1954, 3063.
31. Gänshirt, *Pharmazie*, 1953, **8**, 584.
32. Jurd and Horowitz, *J. org. Chem.*, 1957, **22**, 1618.
33. Personal Communication, T. R. Seshadri, University of Delhi.
34. Oesch and Perkin, *J. chem. Soc.*, 1914, 2350.
35. Power and Salway, *ibid.*, 1910, 231.
36. Personal Communication, G. Tappi, University of Modena, Italy.
37. Heap and Robinson, *J. chem. Soc.*, 1926, 2336.
38. Fukuda, *Bull. Chem. Soc., Japan*, 1928, **3**, 53.
39. Kuhn, Löw and Trischmann, *Ber.*, 1944, 77, 211.
40. Jain, Pankajamani and Seshadri, *J. Sci. Ind., Research* (*India*), 1953, **12**B, 127.
41. Tappi and Karrer, *Helv. chim. Acta*, 1949, **32**, 322.
42. Shimizu, Kirisawa and Ogawa, *J. pharm. Soc., Japan*, 1951, **71**, 875.
43. Deulofeu and Schopflocher, *Gazz. Chim. Ital.*, 1953, **83**, 449.
44. Tappi and Menziani, *ibid.*, 1955, **85**, 694.
45. Vitte and Boussemart, *Bull. Soc. pharm. Bordeaux*, 1952, **90**, 177.
46. Jurd, *Arch. Biochim. Biophys.*, 1956, **63**, 376.
47. Kind and Celentano, *J. org. Chem.*, 1953, **18**, 1473.
48. El Ridi, Khalifa and Mamoon, *J. Pharm. Pharmacol.*, 1956, **8**, 602.
49. Gomm and Nierenstein, *J. Amer. chem. Soc.*, 1931, **53**, 4408.
50. Briggs and Locker, *J. chem. Soc.*, 1951, 3136.
51. Biltz, *Ber.*, 1922, **55**, 1066.

OBSERVATIONS ON THE RELEASE AND TURNOVER RATE OF 5-HYDROXYTRYPTAMINE IN THE GASTROINTESTINAL TRACT

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Received June 8, 1959

Three groups of experiments were carried out on man, dogs and rabbits to strengthen the theory of the intestinal origin of blood 5-hydroxytryptamine (5-HT) and urinary 5-hydroxyindoleacetic acid (5-HIAA), and to contribute to the solution of the problem of the turnover rate of 5-HT in the gastrointestinal mucosa. Serum obtained from blood taken by catheterisation from the hepatic veins contained, in man and dogs, more 5-HT than serum obtained from vena cava blood. Moreover, dogs deprived operatively of the entire gastrointestinal tract presented a sharp drop in the urinary 5-HIAA excretion. It was tentatively calculated, from the data gathered in this study, that half-life of gastrointestinal 5-HT is approximately 6 to 8 hours in dogs, and 7 to 12 hours in man.

The enterochromaffin cells of the gastrointestinal mucosa have been considered for 20 years to be the main site of production and storage of 5-hydroxytryptamine (5-HT, enteramine) in vertebrates[1,2].

Toh[3] was the first to produce direct evidence that blood 5-HT originates from the intestines. He found that in dogs portal blood contained nearly three times more 5-HT than arterial blood, and that there was a spontaneous release of 5-HT from the perfused dog stomach, the output being 0·05 to 0·4 μg. per minute. Bertaccini[4], in this Institute, demonstrated that removal of the large intestine produced in the rat a decrease in the daily urinary excretion of 5-HIAA, that is, a decrease in the daily production and metabolism of 5-HT. Similar results were obtained, quite recently, by Rosenberg and others[5].

According to Erspamer[6] the 5-HT in the rat gastrointestinal tract is completely renewed, in about 8 to 9 hours; according to Udenfriend and Weissbach[7], who employed radioactive precursor amino acids, the biologic half-life of 5-HT in the gastrointestinal mucosa of the rabbit is 11 to 17 hours. This means that the gastrointestinal mucosa of the rat and the rabbit would be capable of synthesising an amount of 5-HT corresponding to that contained in it, in 8 to 9 hours and 22 to 34 hours, respectively.

The three groups of experiments, of which an account is given in this paper, were designed to provide further evidence of the production and release of blood 5-HT from the gastrointestinal tract, and further information about the turnover-rate of gastrointestinal 5-HT.

Experimental and Results

Differences in the 5-HT Content of Serum Obtained from Vena Cava Blood and of Serum Obtained from Hepatic Veins Blood

Blood samples were taken, by catheterisation, from the hepatic veins and from the vena cava, below the inflow of the renal veins, in six

unanaesthetised patients suffering from heart disease and in six unanaesthetised normal dogs. After standing for 4 to 5 hours at room temperature and then overnight in the refrigerator at 3°, the blood samples were centrifuged and the serum treated with 4 volumes of acetone. The 5-HT content of the filtrate was estimated on the atropinised oestrus uterus of the rat[1,8].

During the week preceding the withdrawal of blood, two successive collections of urine were taken, over 24-hour periods, the first from untreated human beings and dogs, the second from subjects treated subcutaneously with 6 mg. (dogs) and 20 mg. (man) of 5-HT creatinine sulphate. In all the urine samples, 5-HIAA was estimated quantitatively by the method of Macfarlane and others[9], to establish the normal urinary output of 5-HIAA in the examined subjects, and the recovery, as excess urinary 5-HIAA, of the injected 5-HT (1 mg. 5-HIAA is equivalent to 0·92 mg. 5-HT).

The daily urinary excretion of 5-HIAA is shown in Tables I and II. The average recovery of exogenous 5-HT, as urinary 5-HIAA, was found to be for man 32 per cent (range 22 to 45 per cent), for dogs 27 per cent (range 20 to 37 per cent). Thus, the ratio of administered 5-HT to recovered 5-HT was 3·1 for men, and 3·7 for dogs. The values found for man agree very well with those reported by Ferrari and Castelli[10].

The daily release and metabolism of 5-HT was tentatively calculated:

(1) from the difference in the 5-HT content of the serum from hepatic venous blood and the serum from vena cava blood, multiplied by the millilitres of serum which pass through the liver during a 24-hour period. Hepatic blood flow is estimated to be approximately 2200 litres/24 hours (= approximately 1100 litres serum/24 hours) for an adult man, and 53 litres/kg./24 hours (= approximately 27 litres serum/kg./24 hours) for the dog[11].

(2) from the normal urinary output of 5-HIAA multiplied by 3·1 in man and 3·7 in dogs. In this calculation it is, of course, arbitrarily assumed that equivalent amounts of both exogenous and endogenous 5-HT give origin to identical amounts of urinary 5-HIAA.

TABLE I

DIFFERENCES IN THE 5-HT CONTENT OF SERA OF DIFFERENT ORIGIN AND DAILY URINARY EXCRETION OF 5-HIAA IN HUMAN PATIENTS. TENTATIVE CALCULATION OF THE DAILY RELEASE AND METABOLISM OF 5-HT

Patient	5-HT content in hepatic (H) and cava (C) serum (μg./ml.)	Excess 5-HT in hepatic serum (μg./ml.)	Calculated daily release of 5-HT (mg.)	Daily urinary excretion of 5-HIAA (mg.)	Calculated daily metabolism of 5-HT (mg.)
D.C.	H 0·086 C 0·06	0·026	28·6	1·13	3·50
B.R.	H 0·19 C 0·158	0·032	35·2	2·60	7·96
N.P.	H 0·13 C 0·104	0·026	28·6	2·26	7·00
D.G.	H 0·083 C 0·078	0·005	5·5	1·85	5·63
P.L.	H 0·11 C 0·095	0·015	16·5	2·90	9·00
A.G.	H 0·098 C 0·078	0·02	22·0	3·50	10·85
	Mean =	0·021	22·7	2·37	7·32

It appears from Tables I and II that in 11 of 12 experiments the blood serum from hepatic veins contained more 5-HT than that from vena cava. The difference ranged from 0·005 to 0·032 μg./ml. for man and from 0·03 to 0·075 μg./ml. for dogs. Toh's[3] data on the release of 5-HT from the dog's gastrointestinal tract were thus confirmed, but the rate of release was found to be considerably less than that stated by this investigator.

TABLE II

DIFFERENCES IN THE 5-HT CONTENT OF SERA OF DIFFERENT ORIGIN AND DAILY URINARY EXCRETION OF 5-HIAA IN DOGS. TENTATIVE CALCULATION OF THE DAILY RELEASE AND METABOLISM OF 5-HT

Dog	5-HT content in hepatic (H) and cava (C) serum (μg./ml.)	Excess 5-HT in hepatic serum (μg./ml.)	Calculated daily release of 5-HT (mg./10 kg.)	Daily urinary excretion of 5-HIAA (mg./10 kg.)	Calculated daily metabolism of 5-HT (mg./10 kg.)
1	H 0·45 C 0·42	0·03	7·1	0·67	2·5
2	H 0·26 C 0·20	0·06	14·2	0·86	3·2
3	H 0·215 C 0·237	—	—	0·64	2·4
4	H 0·494 C 0·451	0·043	11·6	1·10	4·1
5	H 0·286 C 0·25	0·036	9·7	1·23	4·6
6	H 0·645 C 0·57	0·075	20·2	0·40	1·5
	Mean =	0·05	12·6	0·82	3·05

The daily production of 5-HT appeared to be generally greater, approximately three- to four-fold, if calculated from the difference in the 5-HT content between blood returning from the intestines and vena cava blood, than if calculated from the urinary output of 5-HIAA. We are inclined to consider the values given by the latter method to be more precise. In the first calculation, indeed, the data concerning the 5-HT content, were necessarily obtained by a biological method which is probably less accurate than a chemical method. Moreover, in this calculation it was assumed, presumably incorrectly[12], that release of 5-HT from the enterochromaffin cells is continuous and uniform.

5-HT Content in Blood Plasma of Rabbits Pretreated with Reserpine

To obtain additional and, if possible, more accurate data on the 5-HT release from the gastrointestinal mucosa, a few experiments were made on reserpine-pretreated animals.

Reserpine is known to deplete the 5-HT depots in the organism. Particularly sensitive to the drug are the blood platelets, which not only lose nearly the whole of their 5-HT but become also incapable of absorbing 5-HT from the surrounding medium[13]. Reserpine, on the other hand, does not seem capable of interfering in the biosynthesis of 5-HT[14]. It seems, therefore, conceivable that in animals given reserpine the 5-HT normally released from the gastrointestinal mucosa would appear free in the plasma and that, as a consequence, plasma from blood of intestinal origin would contain more 5-HT than plasma from blood returning from other areas.

Experiments were carried out on three rabbits, weighing 3 to 5 kg. After a blood collection from the ear marginal vein (control serum), the animals were given intravenously 0·2 mg./kg. of reserpine. Forty-eight hours later, a second blood sample was taken from the ear vein (reserpine serum); then the animals were given heparin intravenously and soon after under a light ether anaesthesia, blood samples were withdrawn through polythene catheters from the jugular vein and from a mesenteric vein into siliconised centrifuge tubes. Plasma was separated by centrifugation at 3000 rev./min. for 20 minutes, then treated with 4 volumes of acetone. 5-HT was estimated in the filtrate. Results are shown in Table III.

TABLE III

THE 5-HT CONTENT OF PLASMA OBTAINED FROM MESENTERIC VENOUS BLOOD AND FROM JUGULAR BLOOD OF RABBITS PRETREATED WITH RESERPINE

	5-HT content (μg./ml.) in			
	Control serum	Reserpine serum	Reserpine mesenteric plasma	Reserpine jugular plasma
Rabbit 1	3·1	0·025	<0·02	<0·02
,, 2	2·2	0·2	<0·02	<0·02
,, 3	4·5	0·12	<0·02	<0·02

It may be seen that under our experimental conditions no difference could be found in the 5-HT content of plasma from mesenteric vein blood and plasma from jugular vein blood. It follows that in the rabbit the release of 5-HT by the gastrointestinal mucosa is less than 0·02 μg./ml. The rat uterus preparation is unsuitable for an accurate estimation of lower concentrations of 5-HT. We are now repeating our experiments in rabbits and dogs given reserpine, using the more sensitive Vane's method[15].

Serum 5-HT and Urinary 5-HIAA Following Removal of the Gastrointestinal Tract in Dogs

Two dogs were subjected, under pentobarbitone anaesthesia, to removal of the entire gastrointestinal tract, the spleen and the pancreas. The operation was preceded by the withdrawal of a sample of arterial blood and an estimation of 5-HIAA in urine collected over a 24-hour period. After the operation the dogs were given antibiotics and sufficient amounts of physiological saline, in part intravenously and in part subcutaneously. One dog died after 48 hours, the second after 25 hours. During the survival period urine was carefully collected and blood samples drawn from the femoral artery after 20 and 40 hours. The results are set down in Table IV. This Table shows two interesting facts.

Removal of the gastrointestinal tract, that is, of the enterochromaffin cell system, does not apparently modify, during the brief survival period, the 5-HT content of serum. Without excluding that a real decrease in the 5-HT content of blood may be in part masked by a concomitant *inspissatio sanguinis*, we consider that the observed fact is strongly indicative of the tenacity with which platelets retain their 5-HT or at least a

remarkable aliquot of it. This agrees well with the calculated half-life of platelet 5-HT (33 to 48 hours[7]).

The 5-HIAA content of urine declines rapidly after the operation. The urine of the first 24 hours contains about 20 to 25 per cent of the 5-HIAA in normal urine; later on, this metabolite seems to disappear completely.

TABLE IV

SERUM 5-HT LEVELS AND URINARY 5-HIAA EXCRETION IN DOGS AFTER REMOVAL OF THE GASTROINTESTINAL TRACT

	Serum 5-HT µg./ml.	Urine volume ml./24 hours	Urinary 5-HIAA µg./24 hours
Dog 1 (12 kg.)—			
Before operation	0·42	756	674
0–24 hours after operation ..	0·47	230	170
24–48 hours after operation ..	0·30	400	? (<40)
Dog 2 (14·5 kg.)—			
Before operation	0·20	1013	860
0–25 hours after operation ..	0·23	200	180

We are perfectly aware of the extreme caution with which these results should be interpreted. These are, however, in good accordance with those obtained by other research workers[4,5] in very extensive studies.

DISCUSSION

The experiments described in this paper support the theory that blood 5-HT originates from the gastrointestinal mucosa. Moreover, this mucosa seems to be, on the basis of the available data, the only source of all the extracerebral 5-HT and, as a consequence, of all the urinary 5-HIAA. A possible exception is found, among the mammals, in some rodents (rats and mice) in which the mast cells also may in theory contribute to the biosynthesis of blood 5-HT and urinary 5-HIAA. Experiments are in progress in this laboratory with the purpose of throwing some light on the problem of the part played by the rat mast cells in the production of the 5-HT found in blood and the 5-HIAA found in urine.

The data here presented may also help in the elucidation of the problem of the turnover rate of gastrointestinal 5-HT in mammals, more precisely in that of the man and the dog.

If we accept that, for a dog weighing 10 kg., the 0·82 mg. of 5-HIAA excreted over a 24-hour period, originates from 3 mg. of 5-HT, then we ought to conclude that a quantity of 5-HT corresponding to that contained in the entire gastrointestinal tract (approximately 1·5 to 2 mg.) is synthesised every 12 to 16 hours. The half-life of gastrointestinal 5-HT would be approximately 6 to 8 hours.

For man, assuming that the 1500 g. of gastrointestinal tract contains an average of 3 to 5 μg. of 5-HT per g. of fresh tissue[8], the total content of the gastrointestinal tract would be 4·5 to 7·5 mg. 5-HT. If the figure for the daily metabolism of 5-HT, calculated from the urinary 5-HIAA,

is reasonably accurate (7·3 mg.), then a quantity of 5-HT corresponding to that contained in the entire gastrointestinal tract would be synthesised every 14 to 24 hours, that is, the half-life of gastrointestinal 5-HT would be approximately 7 to 12 hours.

It may be seen that the above values agree fairly well with those obtained by Udenfriend and Weissbach[7], who used radioactive tryptophan and 5-hydroxytryptophan and found that in the rabbit the half-life of intestinal 5-HT was 11 hours.

It should be kept in mind, however, that there is increasing evidence demonstrating that the biosynthetic possibilities of the enterochromaffin cells are far from being completely exhausted under normal conditions. Some experimental results, for example, seem to indicate that the turnover rate of gastrointestinal 5-HT may be conspicuously increased when excess dietary tryptophan is offered to the enterochromaffin cells. In fact, Lauer and others[16] and Kopin[17] found that human subjects doubled their urinary excretion of 5-HIAA in the 6-hour period after oral administration of 5 g. of L-tryptophan.

Acknowledgement. This work was supported by a grant from the Rockefeller Foundation, New York.

References

1. Erspamer, *Arch. exper. Path. u. Pharmakol.*, 1940, **196**, 343 and 1942, **200**, 43.
2. Erspamer, *Pharmacol. Rev.*, 1954, **6**, 425.
3. Toh, *J. Physiol.*, 1954, **126**, 248.
4. Bertaccini, *Naturwissenschaften*, 1958, **45**, 548.
5. Rosenberg, Davis, Moran and Zimmermann, *Fed. Proc.*, 1959, **18**, 503.
6. Erspamer, *J. Physiol.*, 1955, **127**, 118.
7. Udenfriend and Weissbach, *Proc. Soc. exp. Biol. N.Y.*, 1958, **97**, 748.
8. Erspamer, *Rendiconti scient. Farmitalia*, 1954, **1**, 1.
9. Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus and Smith, *Scottish med. J.*, 1956 **1**, 148.
10. Ferrari and Castelli, *Rass. Fisiopatol. clin. terap.*, 1954, **26**, 689.
11. Lovatt Evans, *Principles of Human Physiology*, 12th Ed., Churchill, London, 1956.
12. Johnsen, Smith and Simon, *Clin. Research*, 1958, **6**, 268.
13. Hardisty, Ingram and Stacey, *Experientia*, 1956, **12**, 424.
14. Erspamer and Ciceri, *ibid.*, 1957, **13**, 87.
15. Vane, *Brit. J. Pharmacol.*, 1957, **12**, 344.
16. Lauer, Inskip, Bernson and Zeller, *Arch. Neurol. Psych.*, 1958, **80**, 122.
17. Kopin, *Science*, 1959, **129**, 835.

THE SURFACE ACTIVITY OF 1,16-HEXADECANE DISODIUM SULPHATE AT THE AIR : WATER INTERFACE

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Received June 10, 1959

The surface tensions of 1,16-hexadecane disodium sulphate in 0·001, 0·2 and 1·0M $l.^{-1}$ sodium chloride solutions have been determined by the Wilhelmy plate method. Considerable ageing effects were noted. The minimum molecular areas calculated were 95, 88 and 86 sq. Å respectively in the three salt solutions. The lowering of the surface tension was not as great as that caused by sodium dodecyl sulphate, and the hydrocarbon chain linking the two head groups in 1,16-hexadecane disodium sulphate appeared to prevent very close packing in the surface layer.

The introduction of a second polar group, remote from the first, into the structure of normal soaps, has been found[1] to decrease the size of the aggregates formed. Studies with dipotassium 1,16-hexadecanedioate disclosed a concentration limit at 10 to 11 mM $l.^{-1}$, where the solute aggregated to give dimers.

It is of interest to study the surface tensions of solutions of this type of material; to ensure freedom from hydrolysis in dilute solution 1 : 16-hexadecane disodium sulphate (HDS) was chosen.

Experimental

Materials. 5 g. (0·019 mole) of 1,16-hexadecanediol was dissolved in 20 ml. glacial acetic acid, and 4·6 g. (0·038 mole) of chlorosulphonic acid added slowly to the solution, which was stirred and kept cool. After allowing to warm up to room temperature and stirring for a further thirty minutes, the mixture was neutralised with solid $NaHCO_3$. The crude HDS was extracted with two 50 ml. portions of boiling 90 per cent ethanol; on cooling the extract crude HDS crystallised. The solid was recrystallised three times from water, dried, extracted for 96 hours with ether to remove traces of glycol, and recrystallised three times from conductivity water. Analysis for sulphur gave 13·92 per cent (theoretical 13·89 per cent). Commercial methanol was fractionally distilled and the product had b.p. 64·7°, n_D^{15} 1·3303. (Timmermans[2] gives b.p. 64·75°, n_D^{15} 1·33057.) Ion exchanged water was distilled from a seasoned still. The sodium chloride used was Analar quality.

Surface tensions were measured by the Wilhelmy plate method[3,4]. A thin platinum plate was suspended from one arm of a chainomatic balance reading to 0·1 mg.; the balance rested on a platform movable in the vertical direction by a rack and pinion device. The exact point where

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the lower edge of the plate just touched the liquid surface could be determined by slowly lowering the balance platform. The weight required (G) to raise the plate from the interior of the liquid to this point in the surface was determined. To ensure zero contact angle, all measurements were made by lifting the plate from the interior of the liquid, also the liquid was placed in a tall flask which was almost submerged in a thermostat. This ensured a saturated atmosphere, which prevented evaporation from the line of contact between liquid and plate. All glassware and the plate were cleaned in chromic acid before use.

The surface tension, γ, was calculated from

$$\gamma = g \frac{(G - W)}{l}$$

where W was the weight of the dry plate in air, and l was its perimeter. All measurements were made at 25° ± 0·05°.

Results

A check of the apparatus was made by measuring the surface tensions of water and methanol. Water at 25° gave $\gamma = 71{\cdot}92$ dynes cm.$^{-1}$ (71·97); at 20°, $\gamma = 72{\cdot}64$ dynes cm.$^{-1}$ (72·65). Methanol at 25°, $\gamma = 22{\cdot}15$ dynes cm.$^{-1}$ (22·18)[2]. These results were in good agreement with literature figures, which are given in brackets.

Ageing effects were observed with all solutions of HDS. The Wilhelmy plate method proved to be particularly useful in studying these changing surface tensions. Some examples of surface ageing are given in Table I.

The surface tensions of the salt solutions alone were found to be constant, within experimental error, over the time-period studied. For HDS solutions, a constant value of the surface tension was generally obtained in 1 to 2 hours; equilibrium appeared to be reached faster in the more concentrated salt solutions.

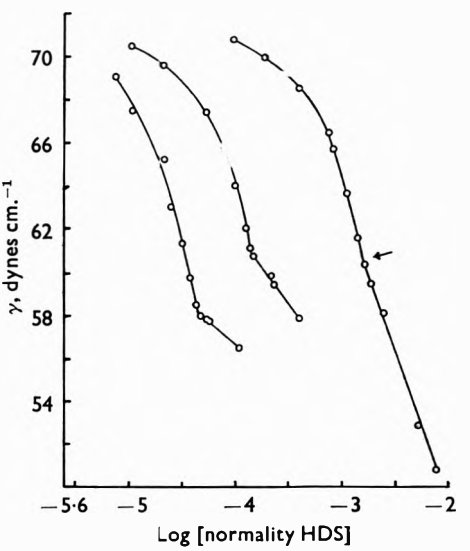

FIG. 1. Plot of surface tension, γ, against log (normality HDS).

The results for the variation of surface tension with concentration are shown in Figure 1. All curves showed a break at the critical micelle concentration (CMC), the break becoming more pronounced as the salt concentration was increased. No minimum was observed in any of the

curves, demonstrating the purity of the HDS sample. The values of the CMC's were:

NaCl concentration, M $l.^{-1}$ ..	1·0	0·2	0·001
CMC, mM $l.^{-1}$	0·0215	0·0700	0·79

TABLE I

SURFACE TENSION AGEING EFFECTS WITH SOLUTIONS OF HDS

Surface tensions in:—

	0·00M $l.^{-1}$ NaCl		0·2M $l.^{-1}$ NaCl		1·0M $l.^{-1}$ NaCl	
Time (mins.)	Alone	÷0·001386N HDS	Alone	÷0·0001243N HDS	Alone	÷0·00003156N HDS
0	$71·9_0$	67·2	$71·9_8$	65·2	$73·2_0$	63·0
5		66·4		64·7		62·5
10		65·8		64·2		62·2
20		64·5		63·8		61·8
30		63·5		63·4		61·6
40		62·8		63·2		61·4
50		—		63·1		61·3
60	$71·8_8$	62·2	$72·0_2$	62·8	$73·3_1$	61·3
80		61·9		62·4		—
100	$71·9_4$	61·7	$72·0_6$	61·1	$73·3_0$	61·4
120		61·6		61·0		—
140		61·7		61·1		—
180	$71·9_1$	61·6	$72·0_4$	61·1	$73·2_7$	61·3
240	$71·9_3$	61·6	$72·0_5$	61·1	$73·2_4$	61·3

DISCUSSION

The surface excess, τ_2, of a solute, at constant temperature and pressure, when the surface excess of the solvent is zero, has been shown to be related to the change in surface tension, $d\gamma$, and to the chemical potential, μ_2, of the solute by:

$$-d\gamma = \tau_2 d\mu_2 \quad .. \quad .. \quad .. \quad (1)$$

and as $d\mu_2 = \mathrm{RT}dlna_2$, where a_2 is the activity of the solute,

$$-d\gamma = \mathrm{RT}\tau_2 dlna_2 \quad .. \quad .. \quad .. \quad (1a)$$

For dilute solutions this equation approximates to

$$-d\gamma = \mathrm{RT}\tau_2 dlnc \quad .. \quad .. \quad .. \quad (2)$$

where c is the molar concentration.

For electrolytes the situation is more complicated. In the case of a 1:1 electrolyte (for example, sodium dodecyl sulphate Na^+DS^-) it has been suggested that the Gibb's equation should be written[5,6] as:

$$-d\gamma = 2\mathrm{RT}\tau_2 dlna_{\pm} \quad .. \quad .. \quad .. \quad (3)$$

where $a_{\pm}$ is the mean activity, as for a completely dissociated univalent electrolyte

$$d\mu_2 = 2\mathrm{RT}dlna_{\pm}$$

Results from radioactive isotope[7] and spread monolayer experiments[8] indicate that equation (3) does not always hold.

If the detergent is considered in the presence of an electrolyte with which it has one ion in common, certain simplifying assumptions[9] can be made, which lead to the use of equation (1*a*) for determining the surface excess. The results for τ_2 now agree with those from the radio-isotope and spread monolayer experiments.

In the present study, equation (2) is applied by considering each end of the molecule separately. The concentration term in (2) is taken as the normality, and from τ_2 the area of half the molecule can be obtained. The total area : molecule (A) is plotted against the surface pressure (π) in Figure 2 (1), (ii) and (iii).

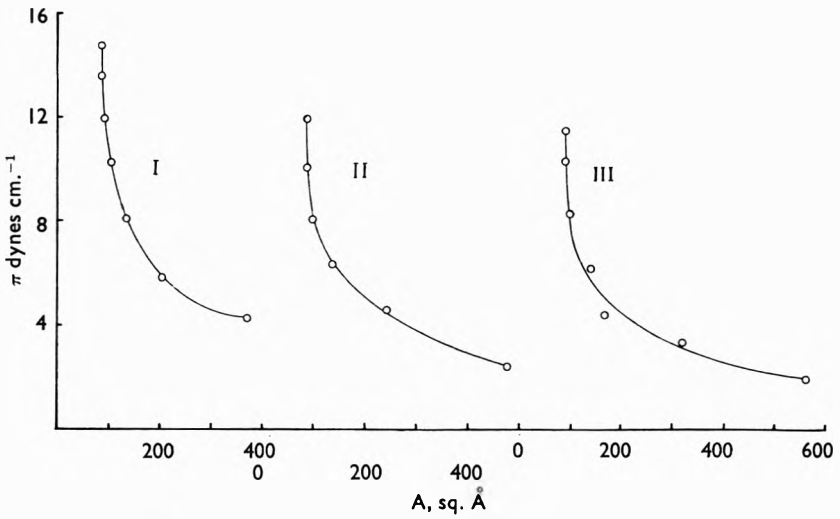

FIG. 2. Force (π)—area (A) curves for HDS in different sodium chloride concentrations. I, 1·0; II, 0·2; III, 0·001 M l.$^{-1}$ NaCl.

All π-A plots follow the same general pattern: there is a slow increase of π with decreasing area, till at about 120 to 150 Å, the surface pressure begins to increase sharply. This increase presumably corresponds to the point where the film begins to be close packed. The limiting areas at the highest surface pressures are given in Table II.

TABLE II

MOLECULAR AREAS AT HIGH SURFACE PRESSURES

NaCl concentration, M l.$^{-1}$	1·0	0·2	0·001
HDS at 25°, sq. Å	86	88	95
NaDS at 20°, sq. Å	27	33	59

Figures for sodium dodecyl sulphate are included for comparison.

Figures for sodium dodecyl sulphate are included for comparison.

The structure of the HDS film at the air : water interface is suggested to be the one in which both polar head groups are immersed in the water, and the hydrocarbon chain pressed up above the surface. In 1·0 and 0·2M l.$^{-1}$ sodium chloride solutions, the area : molecule for HDS is more than double that for NaDS. This indicates that the hydrocarbon chain prevents as close a packing as is given by NaDS. It would appear that the degree to which the hydrocarbon chain can be bent

effectively limits the area : molecule. Spread monolayers of NaDS on 75 per cent ammonium sulphate solutions gave a limiting area of approximately 20 sq. Å[9], and this value approaches the maximum amount to which NaDS can be compressed. It is obvious from these figures that the two head groups of HDS are not forced close together. It will be noted (Table II) that for NaDS a change of 22 sq. Å occurs on increasing the salt concentration from 0·001 to 1·0M $l.^{-1}$. For the same change in salt concentrations, HDS shows a change in the area : molecule of 9 sq. Å.

A crude idea of the shape of the HDS molecule at maximum compression can be gained by studying models. The fully extended molecule has an area of 120 sq. Å. A moderate amount of bending of the chain reduces the area : molecule to 80 to 90 sq. Å and a certain amount of strain will be imposed on the chain in a configuration of this type. Bending the chain until the two head groups touch imposes a great deal of strain, and gives an area : molecule of 60 to 70 sq. Å. The area : molecule obtained experimentally at the highest salt concentration (86 sq. Å) is compatible with a slightly bent chain.

The amount of surface tension lowering given by HDS is not as great as that given by NaDS. It is to be expected that fewer head groups per unit area can be forced into the surface by HDS than by NaDS. At 25° the CMC of NaDS is 0·785 mM $l.^{-1}$ in 0·2M $l.^{-1}$ sodium chloride, while in the same salt solution HDS has a CMC of 0·0700 mM $l.^{-1}$.

No ageing effects have been reported[10] for NaDS in salt solutions after the first few minutes. The ageing with HDS may be due to either the bending of the hydrocarbon chain, or difficulty in moving a molecule from the bulk to the surface.

Acknowledgements. I should like to thank Dr. L. Saunders for advice and encouragement, and Dr. E. P. Taylor of Allen and Hanbury's Ltd., Ware, for the gift of the 1,16-hexadecanediol.

References

1. Elworthy, *J. Pharm. Pharmacol.*, 1959, **11**, 557.
2. Timmermans, *Physico-Chemical Constants of Pure Organic Compounds*, Elsevier, London, 1950, p. 305.
3. Wilhelmy, *Ann. Physik.*, 1863, **119**, 177.
4. Harkins and Anderson, *J. Amer. chem. Soc.*, 1937, **59**, 2189.
5. Brady, *J. Phys. Chem.*, 1949, **53**, 56.
6. Cockbain and McMullen, *Trans. Farad. Soc.*, 1951, **47**, 322.
7. Salley, Weith, Argyle and Dixon, *Proc. Roy. Soc.*, 1950, *A* **203**, 42.
8. Brady, *J. Coll. Sci.*, 1949, **4**, 417.
9. Pethica, *Trans. Farad. Soc.*, 1954, **50**, 413.
10. Matijevic and Pethica, *ibid.*, 1958, **54**, 1382.

MICROCHEMICAL IDENTIFICATION OF SOME ATROPINE-LIKE DRUGS

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Received June 30, 1959

Crystal and colour tests are described for 48 atropine-like drugs.

Few plant alkaloids are used for such a wide variety of purposes as atropine, serving as it does as a mydriatic, a spasmolytic, or an anti-secretogogue. In some ways this is a disadvantage, as it is the cause of unpleasant side effects, and for this reason a search has been made for drugs that are more restricted in their action. None of the available substitutes for atropine is entirely specific although in many of them one effect is predominant. Different substances have therefore been developed for use either as mydriatics, or as spasmolytics, while similar substances have found more specialised uses as antitussives, tranquillisers, or hypotensive agents, or for the treatment of Parkinson's disease.

These substances all contain basic nitrogen, many of them being quarternary ammonium compounds. The first to come into use were synthetic tropeines such as homatropine. Subsequently it was found that many simpler substances had similar anti-acetylcholine properties. The majority of these compounds are esters formed by the combination of an amino alcohol with a substituted acetic acid, but the ester linkage is not essential, and both amines and amides with atropine-like properties are known.

It is the purpose of this paper to describe tests for microgram quantities of 48 of these compounds. As it is difficult to discern any clear connection between chemical structure and pharmacological activity, no attempt has been made to classify them under either of these headings. For the sake of convenience, atropine, hyoscyamine, hyoscine and homatropine have been included here, although tests for these alkaloids have been published previously[1].

Experimental Procedure

Crystal Tests

The hanging microdrop technique developed by Clarke and Williams[1] was employed. All substances were dissolved in 1 per cent acetic acid with the exception of butylhyoscine bromide which was dissolved in a mixture of 1 volume of 5N HCl with 2 volumes of ethanol.

The results obtained are given in Table I. Usually two tests are given for each substance, but where there is difficulty in distinguishing between two closely related drugs three tests have been recorded. For some, only a single test could be found, and for three compounds no crystal tests at all. These substances must therefore be identified by their colour reactions.

TABLE I

Substance	Reagent	Crystals	Sensitivity μg.
Adiphenine HCl (Trasentin, 2-diethylaminoethyl diphenylacetate)	— —	— —	— —
Aminopentamide sulphate (Centrine, γ-dimethylamino-αα-diphenylvaleramide)	Potassium tri-iodide 3 Sodium carbonate Platinum bromide	Small plates or needles Oily needles or fans of rods Rosettes of rods	0·05 0·1 0·25
Amolanone HCl (Amethone, γ-diethylamino-*a*-*o*-hydroxyphenyl-*a*-phenylbutyrolactone)	Gold bromide/HCl Gold chloride	Plates and blades O/N Serrated plates	0·1 0·1
Amprotropine dihydrogen phosphate (Syntropan, 3-diethylamino-2,2-dimethylpropyl (±)-tropate)	Lead iodide	*Hexagonal plates	1·0
*apo*Atropine HCl (Atropyltropine)	Platinum chloride Potassium chromate Potassium tri-iodide 2	Small blades and plates often cruciform and serrated Plates, some serrated Shell-like crystals	0·1 0·5 0·25
Atropine acetate	Picric acid Potassium tri-iodide 3	Bunches of plates Rhomboids and wedge-shaped crystals	0·25 0·025
Atropine methonitrate Q (Eumydrin, methylatropine)	Gold chloride Mercuric chloride	Leaflike plates (P) Plates and prisms	0·025 0·1
Atropine-N-oxide HCl Q (Genatropine)	Platinum iodide Potassium bismuth iodide	Rectangular plates, often transparent Rectangular plates	0·025 0·025
Benactyzine HCl (Cafron, Suavital, α-ethylamino-ethyl-benzilate)	—	—	—
Benzhexol HCl (Artane, Pipanol, 1-cyclohexyl-1-phenyl-3-piperidino-1-propanol)	Potassium permanganate Sodium carbonate Gold cyanide	Curved irregular plates Needles, often curving Bunches of plates	0·1 0·1 0·05
Benztropine methanesulphonate (Cogentin, 3-(diphenylmethoxy) tropane)	Potassium chromate Potassium iodide Ammonium thiocyanate	Long plates or blades Fine dendrites Small plates	0·1 0·25 0·25
Caramiphen HCl (Parpanit, 2-diethylaminoethyl-1-phenylcyclopentane-1-carboxylate)	Lead iodide Platinum chloride	Rosettes of branching needles Rhomboidal plates	0·1 0·25
Convenil citrate (Phenesin, phenyl ethylacetic acid β-diethylaminoethyl ester)	Lead iodide	Branching needles, often in rosettes	0·1
Cyclopentolate HCl (Cyclogyl, 2-dimethylaminoethyl-(1-hydroxycyclopentyl)-phenylacetate)	Gold bromide/HCl Platinum chloride	Starlike rosettes Plates or prisms	0·05 0·5
Cycrimine HCl (Pagitane, 1-cyclopentyl-1-phenyl-3-piperidinopropan-1-ol)	Potassium iodide Sodium carbonate Picrolonic acid	Rosettes of rods Masses of rods Branching needles O/N	1·0 0·05 0·25
Dibutoline sulphate Q (Dibuline, (2-dibutylcarbamoyloxyethyl) ethyldimethylammonium)	Gold bromide Mercuric chloride Lead iodide	Masses of small plates (P) Small plates O/N Bunches of blades or needles	0·25 0·05 0·1

Q Quaternary bases.
O/N Overnight.
(P) Best seen under polarised light.
* Crystals form very slowly, and may fail to appear.
† Dilute solutions only.

TABLE I—*continued*

Dicyclomine HCl (Merbentyl, 2-diethylaminoethyl-dicyclohexyl-1-carboxylate)	Potassium iodide Trinitrobenzoic acid	Branching needles Rosettes of plates	0·5 0·5
Diphemanil methylsulphateQ (Diphenatil, Prantal, 4-diphenyl-methylene-1:1-dimethylpiperidinium)	Mercuric chloride Potassium chromate Zinc chloride	Rhomboids Needles or long plates Rosettes of rods	0·1 0·1 0·1
Dipropylline citrate (Profenil, ethyldi-(3-phenylpropyl) amine)	Gold bromide/HCl Lead iodide	Thin transparent plates$^{O/N}$ †Oily rosettes or irregular crystals	0·25 0·025
Eucatropine HCl (4-mandeloyloxy-1,2,2,6-tetramethyl piperidine)	Gold bromide Potassium tri-iodide 3	Bunches of curved plates Elongated plates	0·025 0·25
Hexahydroadiphenine HCl (Trasentin 6H, 2-diethylaminoethyl cyclohexylphenylacetate)	Picrolonic acid	Fine branching needles	0·1
Hexocyclium methylsulphateQ (Tral, 1-(2-cyclohexyl-2-hydroxy-phenethyl-4:4-dimethyl-piperazinium)	Platinum chloride Ammonium thiocyanate	Snowflake rosettes Bunches of rods	0·1 1·0
Homatropine HCl	Gold chloride Potassium bismuth iodide Potassium tri-iodide 3	Plates and prisms Bunches of plates$^{O/N}$ Plates, some serrated	0·0 5 0·05 0·05
Homatropine methobromideQ (Novatropine, methyl homatropinium)	Gold chloride Potassium permanganate Potassium tri-iodide 1	Small needles, often in crosses or snowflakes Long plates Long plates	0·025 0·1 0·05
Hyoscine HBr (Scopolamine, (−)-hyoscine)	Gold bromide/HCl Potassium bismuth iodide	Curving plates Rosettes of plates	0·25 0·05
Hyoscine butobromideQ (Buscopan, Scopolamine-N-butyl bromide)	Potassium tri-iodide 2 Di-sodium phosphate	Bundles of rods Masses of rods	0·25 0·25
Hyoscine methonitrateQ (Skopyl, methoscopolamine)	Gold bromide/HCl Mercuric chloride Picric acid	Plates often in bunches Plates sometimes in rosettes Dense rosettes$^{O/N}$	0·05 0·05 0·25
Hyoscine-N-oxide HBrQ (Genoscopolamine, scopolamine-*N*-oxide)	Potassium permanganate Potassium iodide	Rods or irregular rosettes Rods	0·25 0·25
Hyoscyamine HCl ((−)-hyoscyamine)	Gold bromide/HCl Picric acid Potassium tri-iodide 3	Long plates or needles$^{O, N}$ Rosettes of plates or needles Small plates	0·25 0·25 0·025
Lachesine HClQ ((2-benziloyl oxyethyl) ethyldimethyl ammonium)	Gold bromide/HCl Gold chloride Lead iodide	Irregular rods and needles Rods and needles Small needles in dense bundles	0·025 0·025 0·025
Mepiperphenidol HBrQ (Darstine, 1-(3-hydroxy-5-methyl-4-phenyl-hexyl-1-methylpiperidinium)	Platinum chloride	‡Bunches of long plates	5·0
Methanthelinium metho bromideQ (Banthine, 2-diethylaminoethyl xanthen-9-carboxylate)	Lead iodide Zinc chloride	Bunches of plates$^{O/N}$ Coarse needles	0·25 1·0

TABLE I—*continued*

Substance	Reagent	Crystals	Sensitivity μg.
Octaverine HCl (Spascol, 1-(3,4,5-triethoxyphenyl)-6,7-dimethoxy isoquinoline)	Potassium chromate Sodium carbonate Platinum bromide	Small needles in bundles Plates, mostly rhomboids Bunches of small needles	0·1 0·1 0·1
Oxeladin citrate (Pectamol, 2-(2-diethylaminoethoxy)ethyl diethylphenylacetate)	Lead iodide	Sheaves of fine needles	0·1
Oxyphenonium bromideQ (Antrenyl, 2-diethylaminoethyl α-cyclohexyl-α-phenylglycollate methobromide)	Gold bromide/HCl Gold chloride	Bunches of plates, often serrated Masses of needles and serrated plates	0·025 0·025
Pavatrine HCl (2-diethylaminoethyl-9-fluorene carboxylate)	—	—	—
Penthienate bromideQ (Monodral, 2-diethylaminoethyl-α-cyclopentyl 2-thiopheneglycollate methobromide)	Lead iodide	‡Oily plates (P)	5·0
Pentoxyverine citrate (Carbetapentane, Toclase, 2-(2-diethylaminoethoxy) ethyl-1-phenylcyclopentyl-1-carboxylate)	Lead iodide	Network of hairlike crystals	0·05
Phenactropinium HClQ (Trophenium, N-phenacyl homatropinium)	Potassium iodide Ammonium thiocyanate	Rosettes of elongated plates Dense rosettes	1·0 0·5
Phenglutarimide HCl (Aturbane, 3-phenyl-3-(β-diethylaminoethyl)-2-6-dioxy piperidine)	Platinum iodide	Dense rosettes or hair-like crystals	1·0
Piperidolate HCl (Dactil, 1-ethyl-3-piperidyl diphenylacetate)	Lead iodide	Rosettes of plates	0·25
Pipenzolate methobromideQ (Piptal, N-ethyl-3-piperidyl benzilate)	Lead iodide Platinum chloride	Oily rosettes Oily rosettes	1·0 1·0
Poldine methosulphateQ (Nacton, 2-benziloyloxymethyl-1-methylpyrrolidine)	Gold bromide/HCl Gold chloride Lead iodide	Masses of small blades and plates Masses of blades Small feathery rosettes or sheaves of plates	0·05 0·1 0·1
Procyclidine HCl (Kemadrin, 1-cyclohexyl-1-phenyl-3-pyrolidinopropan-1-ol)	Sodium carbonate Picrolonic acid	Curving needles Clusters of branching needles and oily plates(P)	0·1 0·1
Propantheline bromideQ (Probanthine, 2-di-isopropylaminoethyl xanthen-9-carboxylate methobromide)	Lead iodide Potassium iodide Potassium tri-iodide 1	Small dense rosettes O/N Prisms Small oily plates O/N (P)	0·25 1·0 0·25
Spasmadryl HCl (Diethylaminoethyl-α-β-diphenylpropionate)	Lead iodide Picrolonic acid	Small rosettes and masses of rods Oily rods or plates O/N	0·1 0·1
Tricyclamol HClQ (Lergine, Elorine, (+)-1-(3-cyclohexyl-3-hydroxy-3-phenylpropyl)-1-methylpyrrolidinium)	Potassium chromate Platinum bromide	Small oily rods, sometimes in rosettes Rosettes or sheaves of needles	0·05 0·25
Tridihexethyl iodideQ (Pathilon, Triethyl (3-cyclohexyl-3-hydroxy-3-phenylpropyl)-ammonium)	Potassium chromate	Hedgehogs or rosettes of fine needles	1·0

TABLE II

Substance	Result	Sensitivity μg.
Formaldehyde-sulphuric acid test (Marquis)		
Benactyzine	Orange–green–blue	0·1
Benzhexol	Faint purple	1·0
Benztropine	Yellow	0·025
Caramiphen	Yellow	1·0
Convenil	Faint orange, fading	1·0
Cycrimine	Dull red	1·0
Dibutoline	Red	1·0
Diphemanil	Orange-red	0·1
Dipropyline	Red	0·25
Hexahydroadiphenine	Orange, fading	1·0
Hexocycline	Dull purple	1·0
Lachesine	Orange–green–blue	0·025
Mepiperphenidol bromide	Dull purple–brown	0·25
Methantheline bromide	Blue	0·25
Octaverine	Green-brown	0·1
Oxeladin	Orange	0·5
Pavatrine	Green, fading	0·25
Penthienate bromide	Blue-green–brown	0·25
Pentoxyverine	Slowly orange	1·0
Piperidolate	Faint orange	1·0
Pipenzolate bromide	Orange–green	0·1
Poldine	Orange–green–blue	0·25
Procyclidine	Faint purple	1·0
Propantheline bromide	Green–yellow	0·5
Spasmadryl	Faint orange, fading	1·0
Tricyclamol	Dull purple	1·0
Tridihexethyl iodide	Brown–black-violet–brown	0·5
Ammonium vanadate test		
Adiphenine	Green–blue	0·1
Benactyzine	Orange–olive-brown	0·05
Benzhexol	Dull green	0·25
Benztropine	Yellow	0·1
Cyclopentolate	Brown	0·5
Cycrimine	Red-brown	0·25
Diphemanil	Brown–blue	0·25
Dipropyline	Grey-green	0·25
Hexahydroadiphenine	Faint green	1·0
Hexocycline	Blue-grey	0·25
Lachesine	Orange–olive	0·05
Methantheline bromide	Orange	0·1
Octaverine	Red-brown	0·1
Pavatrine	Brown*	0·1
Penthienate bromide	Purple	0·1
Pentoxyverine	Brown slowly	1·0
Piperidolate	Olive	1·0
Pipenzolate bromide	Orange–green	0·05
Poldine	Orange–green–dull purple	0·05
Procyclidine	Grey-black	0·25
Propantheline bromide	Orange	0·05
Tricyclamol	Grey-purple	0·25
Tridihexethyl iodide	Brown–black-violet	0·5
Ammonium molybdate test		
Amprotropine	Blue	1·0
Benactyzine	Orange–olive-brown	0·25
Benzhexol	Blue-grey	1·0
Benztropine	Yellow	0·025
Cyclopentolate	Blue	1·0
Cycrimine	Brown	0·5
Dibutoline	Faint blue-grey	1·0
Diphemanil	Yellow–green–blue	0·25
Hexocycline	Dull purple	1·0
Homatropine methyl bromide	Orange–blue	0·5
Hyoscine-N-oxide bromide	Blue-green	0·5
Lachesine	Orange–green–blue	0·05

* Green may be seen first.
† Green does not always appear.
‡ Orange forms before the addition of sulphuric acid.

TABLE II—*continued*

Substance	Result	Sensitivity μg.
Ammonium molybdate test—contd.		
Mepiperphenidol bromide	Blue-grey	0·25
Methantheline bromide	Green	0·05
Octaverine	Green	0·1
Oxeladin	Faint blue	1·0
Oxyphenonium bromide	Green	1·0
Pavatrine	Green†	0·1
Penthienate bromide	Purple	0·1
Pipenzolate bromide	Orange-green	0·05
Poldine	Orange-blue	0·05
Procyclidine	Blue-grey	0·5
Propantheline bromide	Green-yellow	0·05
Tricyclamol	Grey	1·0
Tridihexethyl iodide	Green†-blue-grey	0·5
Selenium dioxide test		
Benactyzine	Orange-olive-brown	0·25
Benzhexol	Brown	0·25
Benztropine	Yellow	0·1
Caramiphen	Pale yellow	1·0
Cyclopentolate	Brown	0·5
Cycrimine	Brown	0·5
Dicyclomine	Faint orange	1·0
Diphemanil	Green-grey	0·5
Hexocycline	Brown	0·5
Homatropine methyl bromide	Yellow	0·5
Lachesine	Orange-yellow	0·05
Mepiperphenidol bromide	Faint brown	1·0
Methantheline bromide	Orange	0·5
Octaverine	Green	1·0
Oxyphenonium bromide	Light brown-orange	0·25
Pavatrine	Brown	1·0
Penthienate bromide	Purple-brown	0·05
Pipenzolate bromide	Orange-green	0·25
Poldine	Orange, fading	0·05
Procyclidine	Brown	0·5
Propantheline bromide	Orange	0·25
Tricyclamol	Brown	0·5
Tridihexethyl iodide	(Orange)‡ brown	0·5
Vitali's test		
Adiphenine	—/—/Intense blue-violet	0·025
Amolanone	—/Pale brown/bright yellow	0·25
*apo*Atropine	—/—/Violet	0·1
Atropine	—/—/Violet	0·025
Atropine methonitrate	—/—/Violet	0·025
Atropine-N-oxide	—/—/Violet	0·025
Convenil	—/—/Purple	0·5
Cyclopentolate	—/—/Violet	0·05
Cycrimine	—/Brown/brown	1·0
Dibutoline	—/—/Faint yellow	1·0
Diphemanil	—/—/Black-purple	0·1
Hexahydroadiphenine	—/—/Brown	0·25
Hyoscine	—/—/Violet	0·025
Hyoscine methonitrate	—/—/Violet	0·025
Hyoscine-N-oxide bromide	—/—/Violet	0·025
Hyoscyamine	—/—/Violet	0·025
Methantheline bromide	Faint yellow/—/green	0·5
Octaverine	Red-brown/brown/red-orange	0·1
Pavatrine	Yellow/brown/dull green	0·25
Penthienate bromide	Brown/brown/red-brown	1·0
Piperidolate	—/—/Blue	0·05
Propantheline bromide	Yellow/—/green	0·5
Spasmadryl	—/—/Purple	0·05
Tridihexethyl iodide	Violet, fading/—/—	1·0

Colour Tests

These are made with microdrops on opal glass as described previously[1]. The results obtained are given in Table II and discussed below.

Two further colour reactions may be employed. With the microdiazo test[2], amolanone gives a bright purple colour when coupled with diazotised *p*-nitroaniline, while phenactropinium gives a brown purple, quickly changing to reddish brown. With the paraformaldehyde/phosphoric acid reagent[3] introduced for the identification of solanine, penthienate gives a bright mauve colour, while methantheline and propantheline give a pale orange. It should be noted that the yellow colour originally described[3] as being given by octaverine with the paraformaldehyde/phosphoric acid reagent has been found to be caused by dihydro-octaverine present as an impurity.

Discussion

Microchemical tests for alkaloids and synthetic bases must serve to identify not only the pure substance, but also the same substance extracted from organic material. In the former the base will be combined with some particular acid, while in the latter it will be present as the free base or combined with some acid of the analyst's choice. It is not always realised that colour tests, and occasionally crystal tests, may be modified by the nature of the acid radical present. The outstanding example of this among the compounds dealt with in this paper is tridihexethyl iodide. Here the intense colour of the iodine liberated by the addition of concentrated sulphuric acid masks any colour due to the base. In some of the bromides the liberated hydrobromic acid, which on its own gives a green colour changing to blue with the ammonium molybdate/sulphuric acid reagent, may modify any colour caused by the alkaloid itself.

The tests given in Table II are for the actual drugs containing the acid radicals given in Table I. Investigation of the colours caused by the alkaloid alone was made by treating a solution of the drugs with silver nitrate solution, and removing excess silver with sodium chloride. The precipitated silver halides were filtered off, and tests made on the filtrate. The following differences were noted. Tridihexethyl gave a faint dull purple with all the sulphuric acid reagents, and no colour with Vitali's test. Methyl homatropine and oxyphenonium gave no colours. Mepiperphenidol gave a yellow colour with the Marquis reagent, and no colour with the other reagents. With pipenzolate there was no change in the colours observed, and with methantheline, propantheline and penthienate the only difference was with the Marquis reagent, where the first two substances gave an orange colour, and the last-named a purple. Hyoscine-*N*-oxide gave the same colour (violet) with Vitali's test, but no colour with the ammonium molybdate-sulphuric acid reagent.

The crystal tests described in Table I are not affected by the nature of the acid radical present.

Acknowledgements. I wish to thank Mr. A. W. Baker and Dr. T. Whittet for help in obtaining material. I acknowledge most gratefully gifts of drugs from Messrs. Abbott Laboratories Ltd.; Amido Laboratories; The Alwitt Trading Co. Ltd.; Bayer Products Ltd.; C. L. Bencard Ltd.; Benger Laboratories Ltd.; Bengue & Co. Ltd.; Bristol Laboratories;

British Drug Houses Ltd.; Burroughs Wellcome & Co.; Ciba Laboratories Ltd.; Coates & Cooper Ltd.; Crookes Laboratories Ltd.; Duncan, Flockhart & Co. Ltd.; Geigy Pharmaceutical Co. Ltd.; Glaxo Laboratories Ltd.; Hommel Pharmaceuticals Ltd.; Lederle Laboratories Ltd.; Eli Lilly & Co. Ltd.; Merck, Sharp & Dohme Ltd.; W. S. Merrell & Co.; Pfizer Ltd.; Pharmacia Uppsala; Roche Products Ltd.; Savoury & Moore Ltd.; G. D. Searle & Co. Ltd.; The Upjohn Co.; Vick International Ltd.; Ward, Blenkinsop & Co. Ltd.; and Ward, Casson Ltd. I am also indebted to Mrs. Ann Williams for technical assistance.

References

1. Clarke and Williams, *J. Pharm. Pharmacol.*, 1955, **7**, 255.
2. Clarke, *ibid.*, 1958, **10**, 194.
3. Clarke, *Nature, Lond.*, 1958, **181**, 1152.

BOOK REVIEWS

THE MAST CELLS. By James F. Riley. Pp. x + 182 (including Bibliography and Index). E. and S. Livingstone, Ltd., Edinburgh and London, 1959. 30s.

As long ago as 1877 Paul Ehrlich first described the mast cells but it is only during the last few years that they have assumed an importance in the study of phenomena which have long puzzled biological workers. It is opportune therefore that a book should be written on these cells. This volume contains, in the first place, a review of the early literature on the subject chiefly from a histological standpoint and then an account of the experimental procedures which were used by the author and his colleagues to establish the connexion between the mast cell population and the tissue histamine content. The material is well presented and the book is delightful to read.

In the first 29 pages, Dr. Riley gives a concise picture of the discovery, evolution and distribution of mast cells in normal and pathological states, and endorses Ehrlich's original belief that there is some functional association between these cells and the connective tissue. Sixty years after Ehrlich's discovery, the mast cell had attributed to it its first physiological function when it was found to be the site of formation or storage of heparin. The work carried out since 1950 to indicate that the cell is not only rich in heparin but is equally rich in histamine is described in the last 130 pages of the book.

After illustrating the distribution of mast cells in preparations of cattle and rat, Dr. Riley shows how these cells in the rat undergo profound morphological changes following contact with chemical histamine-liberators. The photomicrographs of the cells in this and the subsequent sections are superbly presented. As the cells disrupt so the histamine is lost from the tissues. But the author is correct to point out that these histamine-liberators do not affect equally the mast cells of all species. Nevertheless, the evidence is overwhelming that the quantity and distribution of histamine is paralleled by the relative density and distribution of tissue mast cells. This is made even more evident when profiles of the skin of different species are examined.

The book is beautifully published on good quality paper and contains an extensive bibliography of 400 references. It is very fitting that Sir Henry Dale has written the foreword as he was a pupil of Ehrlich and has himself contributed so much to the problem of histamine. The data presented will now be available for discussion not only by those who are active workers in this field of research but also by the many others who are interested in naturally occurring substances.

G. B. WEST.

HANDBUCH DER PAPIERCHROMATOGRAPHIE. By I. M. Hais and K. Macek. Vol. I. Grundlagen und Technik. Pp. xxiv + 860 including Index and 242 illustrations). VEB Gustav Fischer Verlag, Jena, 1958. DM.58.40.

As the title of this book (Volume 1: Principles and Techniques) indicates, this is a Handbook of Paper Chromatography (in German), and as such one can detect little in this field of chemical analysis that has not passed under the eyes of the team of its 22 Czech authors. The introduction states that 10,000 references were scanned in the course of preparation, of which 2,300 are quoted.

The structure of the handbook follows conventional lines by giving a historical survey, an extensive theoretical introduction and chapters on equipment,

preparation, developing and identification, the chromatography of radioactive substances, followed by sections on specialised applications and methods for groups of compounds and inorganic substances. Two extensive appendices contain colour reactions and general hints.

An innovation is a theoretical consideration of the relation between R_F value and compound structure preceding each specialised chapter.

The main interest of the book lies in the fact that it comes from a group of scientists in research institutes in a country with which there is normally little contact. Whereas the Anglo-American scientist has little or no knowledge of the Slavonic languages, no such barrier exists for his East European counterpart who, in addition, has free access to English language papers and makes full use of them. The result is that of the references only about one third are from non-English journals, which would make the book more useful in its country of origin than here. Curiosity would have been excited by inclusion of Russian methods but unfortunately only a few references are quoted.

This handbook, which is therefore essentially a compilation, will be found to be useful as a reference book on methods and sources, and one may conclude that a successful attempt is made to cover the entire field of paper chromatographic techniques, which, however, does not excuse occasional verbosity and the inclusion of some too elementary illustrations.

A second volume is promised which will deal more thoroughly with specific methods as described in the literature.

B. REIFF.

LETTERS TO THE EDITOR

Catechols and Tryptamines in the "matoke" Banana (*Musa paradisiaca*)

SIR,—Varieties of *Musa paradisiaca* are grown abundantly in Uganda, where the pulp of the unripe fruit, known as "matoke", when boiled forms the staple diet of the Buganda tribe. As relatively large amounts of catechols and tryptamines have been found in the varieties of banana eaten in Britain, it seemed important to find out if the "matoke" banana also contains these amines.

A sample of the bananas was kindly supplied by Professor Heller of Bristol. The fruit had been stored in deep freeze since its arrival from East Africa by refrigerated transport. The sample was sent to Dundee in a vacuum flask containing solid CO_2, where it was again stored in deep freeze until extraction of the amines. Catechol amines were extracted from half the sample with 0·01 HCl; the other half was extracted with acetone for tryptamines. The extracts were reduced to small volume by distillation *in vacuo*. Catechols were assayed on the cat blood pressure, 5-hydroxytryptamine (5-HT) on the isolated rat uterus.

	Catechols (μg./g.)		5-Hydroxytryptamine (μg./g.)
	Noradrenaline	Adrenaline	
Peel	1·79	nil	1·07
Pulp	2·00	nil	15·2

The results show that the "matoke" banana contains noradrenaline in amount similar to that found in the ordinary banana[1], but there was no adrenaline in the sample studied. There was a considerable amount of 5-HT in the pulp, but much less in the peel. Since the fruit was unripe, this agrees with the observations of West[2] on the ordinary banana. Chromatography of the acetone extract confirmed the presence of 5-HT and showed the absence of tryptamine, tryptophan and 5-hydroxytryptophan.

In view of the large quantities of "matoke" banana pulp eaten by the Africans in Uganda (several pounds weight per day), the amount of 5-HT found might constitute a contributory factor in intestinal disorders.

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September 10, 1959.

REFERENCES

1. Marshall, P. B., *J. Pharm. Pharmacol.*, 1958, **10**, 781.
2. West, G. B., *ibid.*, 1958, **10**, 589.

The Determination of Morphine in Opium

SIR,—A few years ago a modified Mannich method[1], utilising an extraction procedure suggested by Graf[2], was proposed for the determination of morphine in opium. Lately, the method has been critically examined, and some improvements introduced[3].

Most analyses of opium samples present no difficulties by this method. In a few, however, the method fails to give reproducible results.

The original procedure[1] involves exhaustive extraction of an opium, rendered alkaline, with chloroform-*iso*propanol, from which solution the morphine is extracted by shaking with 20 + 15 + 15 ml. of 0·1 N solution of sodium hydroxide. The combined aqueous extracts, neutralised with hydrochloric acid, are concentrated on a steam bath to 30 g. before the morphine is precipitated as the dinitrophenyl ether by adding 30 ml. of a 1 per cent (w/w) solution of fluordinitrobenzene, followed by 5 ml. of 25 per cent solution of ammonia. The isolated morphine-ether is better washed with acetone only, as shown by Garratt and his colleagues[3].

The non-reproducibility mentioned, sudden "drops" by up to 20 per cent being observed, is supposed to be due to the heating of the slightly acid opium extract. Omitting the concentration of the opium extract, using the following simplified procedure, the reproducibility has been restored. The morphine is extracted as the phenolate from the chloroform-*iso*propanol solution by shaking with 15 + 10 + 10 ml. of 0·1 N solution of sodium hydroxide. Each time the separator is rinsed with 5 ml. of water. The bulked aqueous extracts and washings (50 ml.) are buffered to pH 6 by dissolving 0·50 g. of citric acid, and the morphine-ether is precipitated with 50 ml. of the reagent solution. Continue as above.

As 100 ml. of a 50 per cent (v/v) mixture of acetone and water dissolves 8 mg. of the morphine dinitrophenyl ether, the results obtained by the new procedure are estimated to be about 5 per cent low, calculated with reference to an opium containing 10 per cent of morphine. It is of interest, then, to report some results from morphine determinations by this procedure, as well as by the method adopted by the International Pharmacopoeia, Ed. I.

TABLE I

ANALYSIS OF OPIUM

Sample of opium	Per cent anhydrous morphine			
	Proposed method		Ph. I. Ed. I.	
Number 1	11·05*		10·8	10·8
			10·8	10·8
Number 2 (Yugoslavian)	17·8	17·8	17·3	
	17·8		17·3	
Number 3	15·1	15·4	14·1	13·8
	15·4	15·5	14·1	
Number 4 (Yugoslavian)	11·3	11·6	11·2	
	11·4	11·6	11·0	
	11·5	11·5		
	11·4	11·5		
Number 5 (Yugoslavian)	16·9	17·3	16·5	
	17·1	17·5	16·5	
	17·1	17·4		
	17·0	17·4		

* Mean of 11 determinations standard deviation 0·16.

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September 12, 1959.

REFERENCES

1. Baerheim Svendsen and Drottning Aarnes, *Sci. Pharm.*, 1955, **23**, 18.
2. Graf, *Dtsch. Apoth. Ztg.*, 1951, **91**, 797.
3. Garratt, Johnson and Lloyd, *J. Pharm. Pharmacol.*, 1957, **9**, 914.