TESTS FOR THE STERILITY OF PHARMACEUTICAL PREPARATIONS*

THE DESIGN AND INTERPRETATION OF STERILITY TESTS

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Sterilisation was defined in the United States Pharmacopœia (XIIIth Revision) as "the destruction of all living organisms and their spores in, or their removal from, materials". The word is similarly defined elsewhere, for example, in the British Pharmaceutical Codex 1954 and the Japanese Pharmacopœia 1951. By inference sterility is the state of being free from living organisms and a sterile product is one entirely free from living organisms of all types. This concept is simple enough but unfortunately it is unreal, being incapable of experimental verification. A product is generally regarded as sterile because it has been subjected to a process believed to destroy or remove all micro-organisms and may therefore be expected to pass any sterility tests specified by national pharmacopœias or other authorities. Practical experience however has shown that neither exposure to a process of sterilisation nor passing sterility tests can give absolute certainty of sterility, in the sense of complete absence of living organisms. The most that can be claimed is a probability that the product is sterile although that probability may be very high as, for example, when a needle has been heated until it is red hot or saturated steam under pressure has been properly applied. The designation sterile is therefore to a certain extent arbitrary and official restrictions are generally placed on its use.

A number of pharmacopœias describe processes of sterilisation. Some of these are admitted to be uncertain because it is known that bacterial spores may survive boiling or Tyndallisation, for example. Other processes are considered completely effective: exposure to saturated steam at temperatures of 115° C. and above, heating in aqueous liquids containing 0·2 per cent. chlorocresol or 0·002 per cent. phenylmercuric nitrate at 98–100° C. and heating in a hot air oven at temperatures not less than 150° C. are usually regarded in this light if applied for a sufficient period of time. In fact, none of these can be so accepted. I have had in my possession an organism whose spores regularly survived autoclaving at 115° C. and more for 30 minutes, and Davies and Davison[1] and Davison[2] using heavy inocula of *Bacillus cereus* found that heating with either of the bactericides mentioned above failed to give sterility. The difficulty of controlling physical conditions in the usual type of hot air oven[3] makes dry heat sterilisation a notoriously uncertain procedure and Bowie[4,5] has criticised many of the pressure steam sterilizers at present in use, though not all his criticisms would receive general support[6].

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Admittedly there is no convincing evidence that pathogenic organisms have survived these official processes when they have been efficiently carried out and the survival of non-pathogenic sporing bacteria of abnormally high thermal resistance added in numbers far exceeding those met with in practice is not of great pharmaceutical significance. Nevertheless, the point must be accepted that exposure to one of these processes gives no certainty of sterility although it may give a high degree of probability of sterility.

As certainty cannot be obtained by the knowledge that an officially recognised process has been applied then can it be found by applying sterility tests to the products? The answer is of course an affirmative, but a limited affirmative. It is with these limitations that I hope to deal briefly. Although throughout the paper stress is laid on the limitations of tests for sterility, nevertheless it must be borne in mind that it is on such tests that the whole structure of knowledge of sterilising processes has been built up and when we consider that a process is sufficiently certain in its result to require no subsequent test we are in fact basing our stand on the results of large numbers of tests carried out theretofore.

A sterility test is an experiment carried out with the object of ascertaining certain facts about the flora and fauna of the system under examination. The potential yield of information is limited by the patent impossibility of testing for the whole wide range of possible organisms, and no test at all can be carried out without alteration or destruction of the system under examination. It is therefore impossible to say with certainty that the contents of each container are sterile, or even that they were sterile.

Clearly, what is known as a test for sterility is nothing of the sort, though we might relegate the operative word to inverted commas and designate the procedure as a test for "sterility" i.e., sterility within the meaning of the Act, regulation or pharmacopœia. What, in fact, we carry out is a test for certain contaminant organisms.

Bearing this in mind, let us now look more closely at such a test. As it is an attempt to infer the state of the whole from the result of an examination of the part, it is essentially a statistical operation. The organisms which most concern us are the pathogenic bacteria, though in passing we note that the viruses are a not unimportant group of pathogens. So, considering the general case, we take a sample of a sample, place it in a tube and provide those conditions we believe to be most suitable to bring about the vigorous reproduction of micro-organisms. Then within an arbitrary number of days we hope to get a clear cut result in terms of visible growth or no visible growth. On this evidence we must then decide whether the original material from which the first sample was drawn is to be accepted as sterile or condemmed. The evidence is indirect and may indeed be flimsy. We have to make a number of assumptions in assessing its significance and these should always be borne in mind. We assume that the growth arose from the sample tested and not from the culture medium. We check this point by examination of samples of the culture medium. We assume that the growth did not originate from a contaminant introduced during the

manipulation of the test—and Fleming found that mould spores make sport occasionally in the best conducted laboratories; and most pharmacopœias by permitted retesting concede the point. Thus the International (1st Ed.), British (1953), United States XV (1955), Swiss †Supp. 1954 and Belgian (1940) Pharmacopœias budget for the contingency. The French (1949) (General Directions) and the Japanese do not. With the contents of the remaining pharmacopœias I am not familiar, but no doubt the above sample is fairly representative.

Returning to the pharmaceutical aspects of the test for "sterility", we next assume that no visible growth within the arbitrary time period means no microbe in the sample drawn—always and inevitably a very moot point, for if insufficient attention is given to likes and dislikes no growth will certainly occur. We make a limited check of this aspect on a sample of the medium but the highly specialised requirements of many organisms are well known. Many sporing aerobes will not grow in blood media and a good many aerobes, I believe, are not at all happy in Brewer's medium—and how certain can we be that growth will occur in any given period of time. Indeed there is no agreement about the incubation period, times specified varying from one day in the French Pharmacopœia to ten days in the Belgian Pharmacopœia in the case of dressings. But then heat damaged spores have been known to take five months or longer to produce visible growth[7]. I believe the present record is about 18 months[8].

It will not have passed unnoticed that a great deal of sampling is involved in the testing procedure and sampling always spells uncertainty, greater uncertainty than is usually appreciated. Mathematics as well as technology therefore has something to say about the inferences we may draw from our evidence.

To arrive at the meaning of our result let us first consider the purely mathematical point of view and in the manner of mathematicians let us simplify the problem by ruling out all technical doubts. We shall then accept the simple equation that no growth = sterile because for practical purposes we must always accept that. And we shall similarly, for the time being accept the converse that growth = not sterile, in order to find out what inferences we may draw from a given result.

"One swallow does not make a summer" says our proverb, and yet everyone would agree that swallows are as good an indication of summer in this part of the northern hemisphere as any other natural phenomenon. How many swallows then do make a summer? To answer this type of question we have to invoke a probability function . . . as the number of swallows increases, the probability of the presence of the æstival season also increases.

In the same way our confidence in the satisfactory nature of a batch of parenteral solutions grows with each sample tested and found to be "sterile". But how does it grow and when can we feel reasonably sure of

† Note added in press—

Bemerkungen zur Prüfung auf Sterilität das Suppl. II der Ph. Helv. V by Metaxas, Linder and Munzel[22] which has come to hand since writing the above is a most useful commentary on the Swiss test in particular and Sterility Testing in general.

what we want to know? If we find a positive in our test series, the batch is labelled not sterile—but if we do not find such a positive, what may we conclude, for even if we test every container but one in a batch and find them satisfactory, we shall still not be sure of the condition of that final container.

A satisfactory conclusion must be that there is a reasonable likelihood that the batch is safe. The actual value of this likelihood of safety depends on the details of the test carried out which in turn depends on the rules of procedure followed. These vary greatly from country to country, different requirements frequently being laid down according to the nature and size of the container. Thus the Belgian Pharmacopœia requires a test in the case of distributed batches on 3 per cent. of vials up to a maximum of 10, the Japanese Pharmacopœia requires 3 containers to be examined if the lot size is less than 100 and then 1 additional container for every additional 50 or less containers in the batch up to a maximum of 10, the Swiss requirement is more detailed and specifies a maximum of 30 containers for batches in excess of 10,000 and the sample examined to be drawn at random. The U.S.P. on the other hand requires a representative sample of 10 units to be examined in the case of products sterilised by steam under pressure and for all other products a representative sample of 20 units is to be examined. It is clear that all these pharmacopœial directives are concerned solely with control of manufacture, a point to which I shall return later, in particular, the insistence of the U.S.P. on representative samples is noteworthy.

TABLE I

Per cent. infected items in batch:											
0·1	1	2	5	6·5	10	15	20	25	30	40	50
Probability of drawing 20 consecutive sterile items:											
0·98	0·82	0·67	0·36	0·26	0·12	0·039	0·012	0·003	0·0008	0·00004	0·000001

In Britain, the rules of procedure are laid down by the Therapeutic Substances Regulations[9]. These state: "The number of containers for test from every batch shall be 2 per cent. of the containers in the batch or 20 containers whichever is the less, taken at random from the batch, and if so required by the licensing authority, an additional 2 containers for each thousand or part of a thousand after the first". Since information about quality in a homogeneous batch is not related to the size of the batch but to the actual number of samples examined, a point elaborated by Knudsen[18], the efficiency of the test will rise with increasing batch size until the maximum 20 containers are drawn. It is evident that unless contamination of the batch is fairly widespread, it will not be unlikely that 20 successive sterile containers may nevertheless be drawn. In fact if p = the proportion of infected containers in the batch, assuming that an unbiassed sample has been drawn, the probability of obtaining 20 consecutive sterile containers is $(1 - p)^{20}$. A number of values are given in Table I.

These figures, which speak for themselves, show clearly the inability

of a test such as this to detect low levels of contamination. There is, in fact, a 1 in 4 chance that a batch containing 6·7 per cent. of infected containers will give 20 consecutive "steriles" and therefore be passed as "sterile".

When the number of samples drawn is smaller, the test is less stringent and we may consider 500 items as a typical smaller batch. In this case the probability of drawing 10 consecutive sterile items is approximately $(1 - p)^{10}$—this assumes that the probability p remains constant which is not, of course, strictly true. The values given in Table II are then applicable.

TABLE II

Per cent. infected items in batch:											
0·1	1	2	5	6·5	10	15	20	25	30	40	50
Probability of drawing 10 consecutive sterile items:											
0·99	0·90	0·82	0·60	0·51	0·35	0·20	0·11	0·056	0·028	0·006	0·001

So that there is now about a 50:50 chance of failing to detect contamination in a batch containing 6·7 per cent. of infected containers.

But the regulations recognise the possibility of adventitious infection entering during testing with the result that growth is not indicative of an infected container and make provision for the contingency as follows: "If at the examination a growth of micro-organisms is found in any tube, a further sample may be taken from the batch in the quantity specified . . . and the tests may be repeated on the further sample so taken. If on examination of the further sample no micro-organism is found, the sample shall be regarded as having passed the test; but if the same organism is found as was found in the first sample tested, the batch shall be treated as not sterile and the material contained in the batch shall not be issued or used as part of a further batch. If on such examination, a micro-organism is found, but the same micro-organism as was found in the sample first tested is not found, the test may be repeated on a third sample taken from the batch in the quantity aforesaid. If on examination of this sample no micro-organism is found the batch shall be regarded as having passed the test; but if any micro-organism is found the batch shall be treated as not sterile and the material contained in the batch shall not be issued or used as part of a further batch"*.

It is clear that the effect of these additional rules is to make the sterility

TABLE III

Per cent. infected items in batch:									
1	2	5	10	15	20	25	30	40	50
Proportion of such batches which would be passed as sterile:									
99·1	96·7	84	58	36	20	11	5·6	1·2	0·2

* Note added in press—

I am obliged to Mr. C. L. Sargent of the M.O.H. for pointing out that the 1953 amendment[23] adds the following words to the paragraph "unless or until the material has been resterilised and has passed the foregoing tests".

test even less stringent. The values in Table III are quoted from Davies and Fishburn[11] and are based on the assumption that only one infecting organism is present in a batch of 500 items. If more than one infecting organism is present the chance of passing a seriously infected batch is still further increased.

This excursion into the mathematics of induction has been based on simple sampling statistics applied to a rather simplified model of the actual test carried out. The figures apply, provided random samples have been drawn, fairly closely to small containers such as ampoules where the whole of the contents are examined. May we wonder in passing if a test which depends for its interpretation simply on visible growth, gains or loses by subdivision in order to test specifically for less common contaminants?

When we test larger containers an additional uncertainty enters the picture. At low contamination density it will be quite possible to withdraw a sterile sample from an infected container. This is even more true in the case of solids than in the case of liquids where distribution occurs more readily. How in fact should one test a large amount of solid for sterility? Presumably, and I have never been faced with the problem except in the special case of surgical dressings, the procedure would be dictated by the history of the solid e.g., the surface might first be examined, followed by examination of a representative sample obtained after thorough mixing or by core sampler. But whereas the sampling error in the case of the liquid is readily estimated, it is quite unpredictable in the case of the solid.

Sterility control, like any other form of quality control, is achieved not by the inspection operation but by getting at causes. Small samples considered in isolation tell very little about the bulk from which they were drawn, and the Therapeutic Substances Regulations or any other regulations based on the examination of small samples can detect only widespread contamination within a batch, and become increasingly less stringent with decreasing batch size, until with a batch size of 50, for example, we reach the position where it will be the exception and not the rule to throw out a batch containing 50 per cent. of infected containers. Thus many a batch of 500 containers which would fail to pass the test may prove quite acceptable as 10 sub-batches of 50. But if such a test is the best that can be done for the protection of the patient, is it fair to the manufacturer; in fact, is it technologically sound?

We have already noted that most official sampling and examination procedures seem to be designed for the guidance of the manufacturer. They are also frequently used as a basis for subsequent examination by buyers or other interested bodies.

Now when I ask is the test technologically sound, I am bound to recall the warning in the U.S.P. which will bear repetition "Sterility Tests are highly exacting and should be conducted by personnel having had expert training and experience in rigid aseptic technique". Further, the tests must be made under near ideal conditions, as we may be sure that indeed they are in the manufacturers' control laboratories, for while it is a

prime necessity to protect the patient, there is no virtue in throwing away or unnecessarily reprocessing good material due to misleading test results. Real evidence for the rejection of a batch requires the existence of data supplementary to the specified test, e.g., the existence of some form of control test designed to assess the testing conditions and expertise of the operator. Even so, the number of samples tested and the number of control tests performed is likely to be so small that any inference will be subject to a very high degree of uncertainty.

These problems assume an acute form in the testing of sterile surgical dressings and it is in this connection that I have been interested in the problem.

From the point of view of sterilisation, dressings differ in several major respects from other pharmaceutical items. Thus they are highly contaminated with microbes when they enter the steriliser- this is particularly true of cotton wool which after bleaching and washing is dried with hot air, rather than of gauze which is dried on a hot drum, though even gauze will contain on the average about 100 organisms per square inch—they are difficult to manipulate in testing and they are frequently presented for testing in a bacteriologically filthy wrapping. As a result even when the test is carried out under the best conditions of asepsis, appreciable contamination by airborne organisms occurs. For example, Pulvertaft[12], who identified *Cl. tetanii* and *Cl. welchii* in sanitary pads and accouchement sets, found that about 1 in 4 tests was contaminated during testing when working with dressings of undoubted sterility and Savage[13] found 16 positives in a series of 69 routine control tests. The probability of accidental infection during testing clearly depends on the testing conditions and the investigation by Savage stressed the importance of the very local conditions about the dressing in determining this. He concluded that the probability of adventitious contamination could be as high as 0·2. This state of affairs which is not widely appreciated leads to the serious situation when deliveries of dressings which are undoubtedly sound are rejected due to the lack of experience or inadequate technique of the testing bacteriologist. It is not unfair to say that some medical bacteriologists are prone to underestimate the difficulties of sterility testing.

If it be accepted as not abnormal for a substantial proportion of tests to be contaminated by aerial organisms, then it follows that the presence of low levels of contamination cannot be established. Savage was the first worker to clearly recognise this and he extended his argument to saying that since surgical dressings are massively contaminated before sterilisation, failure of the sterilising process may be expected to result generally in overall lack of sterility. (The same argument seems applicable to control of a filtration process in certain cases) i.e., the likelihood of a sterilisation process resulting in a load of wrapped dressings from which it will be possible to draw both sterile and non-sterile samples is small—this supposition has been borne out by many years of practical operation; only very rarely is it possible to obtain such a result as 9 infected samples out of 10. Therefore an inadequately sterilised load

of dressings will result in a complete run of contaminated samples and he stated his users' control test explicitly in the *Brit. med. J.*[14], in the following words: The test shall be made upon 10 dressings (or portions of a dressing) simultaneously and in random order with a control test upon 10 dressings similar to those under examination except that they are certainly known to be sterile as a result of laboratory treatment. Two conditions are essential: (1) not more than 4 of these control cultures may be positive; (2) the size of the test portion must be chosen so that (if the dressing is not sterile) the average number of organisms in each portion is at least 9. When these conditions are satisfied, dressings may be taken as infected when all the test cultures but not more than 4 of the controls, are positive and as sterile when at least one test culture is negative whatever the condition of the controls. If more than four of the controls are positive infection of the dressings cannot be inferred with enough certainty and the whole test is rejected without drawing any conclusions.

For further information about the test the original papers should be consulted and attention is particularly drawn to the difficulty of preparing a suitable set of control dressings.

Thus by showing that the occurrence of even a single negative in a test series is real evidence of sterility. Savage largely circumvented difficulties in the interpretation of results.

In an earlier paper[15] I attempted a mathematical justification of the test based on the assumption of a continuum of testing conditions characterised by a definite infection probability.

The control series of tests is performed in order to check the suitability of testing conditions, but the very limited amount of information available from a control run of only 10 tubes is not generally appreciated. If we regard these tubes as a random sample of overall testing conditions then the mathematical argument goes as follows[16]: if an event is observed to occur a times out of N, an upper limit p to the probability of this event may be assigned such that if the probability were actually p, then an observed number of occurrences as small or smaller than a would occur with a frequency P and corresponding to this probability is the limit of expectation of the number of occurrences in N trials, namely pN. In the above case $a = 4$ and the probabilities are calculated by solution of the equation:

$$P = \frac{10!\,p^4(1-p)^6}{4!\,6!} + \frac{10!\,p^3(1-p)^7}{3!\,7!} + \frac{10!\,p^2(1-p)^8}{2!\,8!} + \frac{10!\,p(1-p)^9}{9!} + (1-p)^{10}$$

See, for example, Davies[17].

The equation may be solved for different values of P and the values in Table IV are taken directly from Fisher and Yates[16] Table VIIIi: Binomial & Poisson Distributions: Limits of Expectation.

Therefore while in the case when 4 positives are observed in the control series the most likely value for the chance of adventitious contamination is 0·4, we can say only that on the average 9 times out of 10 the true values will be less than 0·65 (using the normal probability scale which extends

TABLE IV

No. of positives in 10 controls a	Upper limit of expectation when probability of a or fewer is		
	10 per cent.	2·5 per cent.	0·5 per cent.
5	7·33	8·13	8·72
4	6·46	7·38	8·09
3	5·52	6·52	7·35
2	4·50	5·56	6·48

TABLE V

Probability of chance infection	Probability of rejection of sterile dressings
0·8	0·07 per cent.
0·7	0·13 ,,
0·4	0·007 ,,
0·2	0·00001 ,,

from 0 to 1, in which 1 represents certainty) 39 times out of 40 it will be less than 0·74, but once in 200 tests it will exceed 0·81.

Assuming that the probability of chance infection is the same for a test dressing as for a control dressing and that it remains constant, we may calculate the probability of rejecting sterile dressings. Suppose that we carry out a test in such circumstances that the chance of aerial contamination remains constant at 0·7; there are 3 possible results:

(1) <10 positives in test .. .. Pass
(2) 10 positives in test $<$ 5 in control series.. .. Fail
(3) 10 positives in test $>$ 4 in control series.. .. Repeat test

The probability of each of these categories roughly computed is:

Pass	0·972
Fail	0·0013
Repeat	0·027

Further, 97·2 per cent. of the repeat tests would comply with the requirements for proof of sterility. There is then little error in calculating the probability of rejecting sterile dressings as the product of the probability of the result in the test series and the probability of the result in the control series—see Table V.

It is evident that it varies considerably with the probability of infection with a maximum value of about 0·13 per cent. However, in general, the probability of infection is not known and so the probability of wrong rejection can only be expressed as a function of this unknown value which we may call p.

$$\text{Probability of rejection} = \frac{10!\ 10!}{10!\ 4!\ 6!}\ p^{14}(1-p)^{6}$$

Treating the extreme case (4 control and 10 test cultures positive) by the method of Fisher based on the multinomial expansion, we may calculate the probability of wrong rejection. This is found to be 0·58 per cent. Therefore approximately 1 out of every 200 batches of dressings rejected by this criterion will be wrongly rejected. The corresponding value for the case of 3 positives in the control run is 0·16 per cent. and the value falls to 0·00054 per cent. when there are no positives, i.e., even when 10 positives are found in the test series and none in the control series there is still a definite chance that a sterile batch of dressings may be unjustly condemned. The chance is small—but does not fall into the

same category as the well-known risk alleged by students of thermodynamics that a kettle of water may boil when you put it on a lump of ice. Clearly the manufacturer can regard the test with equanimity.

Considerations such as the above led to the introduction of a modified test for sterility in surgical dressings in the British Pharmaceutical Codex, 1954. This conceded the necessity for a control series as a check on bacteriological conditions and aseptic technique, and required that no positives appear in the control series, and not more than 3 in the test series for acceptance as sterile. Apart from the technical difficulties of the test, the soundness of the numerical requirements may readily be justified in a number of ways. Thus we may look up Fisher and Yates, Table VIIIi; and read off that when no positive occurs in the control series of 10 tubes we may be 90 per cent. certain that the true value estimating contamination is less than 2·06, 97·5 per cent. certain that it is less than 3·09 and 99·5 per cent. certain that it is less than 4·11. This suggests that from the mathematical point of view not more than 4 positives rather than not more than 3 would be doing better justice to the manufacturer. However, the B.P.C. covers the point by stating that conclusions drawn from tests in which the numbers of positives are on or near these limits are subject to a chance of error and the test should be repeated using larger inocula. The non-mathematical may feel that the test can be justified by common sense along the following lines: if no positive appears in the control series of tubes, we may infer that contamination of a tube by chance is a fairly unlikely event (indeed the tabulated figures mentioned above are based on this argument—that a Poisson distribution is involved). That being so, two such events will not frequently occur together, three most infrequently and four is so unlikely to occur that we may infer that chance is not a sufficient explanation.

This type of problem is generally treated statistically by calculation of the function χ^2 which is applied to problems where it is necessary to determine if a given event has occurred with a frequency significantly different from expectation. Calculation of χ^2 for homogeneity implies acceptance of the results of both classifications as their own expectations so to speak[18] and testing them for independence of classification. Thus we have the well-known 2×2 contingency table:

$$\begin{array}{c|c} 0 & 10 \\ \hline 3 & 7 \end{array} \quad \text{where the expected cell values would be} \quad \begin{array}{c|c} 1\tfrac{1}{2} & 8\tfrac{1}{2} \\ \hline 1\tfrac{1}{2} & 8\tfrac{1}{2} \end{array}$$

Unfortunately in the case under consideration the numbers are very small and unbalanced so that this approach is not possible even making Yate's correction for continuity. However, Fisher and Yates, Table VIII, suggests that the value of χ_c obtained in the case of the distribution: $\begin{array}{c|c} 0 & 10 \\ \hline 4 & 6 \end{array}$ which is the one which mainly concerns us, does not reach the 1 in 40 level of significance, in other words again, it is not, mathematically, a wholly satisfactory criterion. That is not to say that the mathematician asserts that a given result is indicative of sterility or otherwise. He can

merely comment on the adequacy of the evidence and the above finding is confirmed by making the exact calculation of the probability of heterogeneity by the method of Fisher[19] which gives the value:

$$p = \frac{4!\,16!\,10!\,10!}{20!}\left(\frac{1}{10!\,4!\,6!}\right) = 0{\cdot}043.$$

The precision of the test could be improved by taking a greater number of samples, but it is generally felt that the examination of 20 samples of surgical dressings is enough for anyone—the mathematical assumption of constant testing conditions might be invalidated in a larger series due to human nature alone, which also may provide some corrective to the strictness of the test, in that unless testing conditions can be made really first rate, there is going to be a great deal of repetitive testing. On the other hand, if the control series of dressings has not been properly prepared, particularly in the case of dressings whose wrappings have become dusty or dirty in store, the test will be heavily biassed and the basis of the above mathematics will be invalidated.

So we see that it is very difficult to achieve full control of surgical dressing sterilisation by means of orthodox sterility testing, though in the manufacturer's laboratory the yield of information is greater than has been suggested above. Thus the assembly of control tests plotted as a control chart together with plate counts give a useful guide to testing conditions, and the identification of organisms found gives further useful information about sources of contamination. In fact because of the invariable presence of sporing organisms in surgical dressings, I have suggested that under many circumstances pasteurisation of the medium immediately after inoculation would result in much more information per test. This procedure was carried out for some time as a check on other methods of control. And while on the subject of refinements of technique, I should like to draw attention to the B.P.C. instruction to test with larger inocula in the cases of doubt. In many instances this is sounder than the more usual instruction to draw and test a larger number of samples since it increases the probability of finding contaminants without appreciably affecting the adventitious contamination rate.

In practice, in the company with which I was associated, primary control of sterilisation was based on the examination of earth packets containing thermoresistant spores which were strategically sited about the load. This is the method of control required by the Belgian Pharmacopœia which suggests however that *B. subtilis* is a suitable organism. Berry has shown that cultures of that organism vary very widely in their resistance to heat and the same is probably true of most other pure cultures. So that the German test material, based on the work of Konrich[20], dried and sieved garden earth with a sufficient content of native spores to resist steam at 120° C. for 5 minutes, is much more satisfactory.

Not any earth will do. Samples from some parts of the factory grounds were useless, but samples from one particular spot have been in constant use now for 20 years and have found their way into a number of other institutions as test organisms.

The actual organisms which yields the resistant spores has been described by Savage[21] as a slender rod which grows slowly to form chains. Despite its good record, the thermal death-point is kept under constant observation. About $\frac{1}{4}$–1 g. of earth is used per test packet and this is subsequently incubated in 15 ml. of B.P. aerobic medium. Cases of doubtful growth are resolved by microscope since subculturing is frequently unsuccessful due to the necessity for a growth factor present in the earth.

There seems no reason why this method of control should not be more widely utilised since, if a large inoculum of this organism, which is so much more resistant than pathogens such as *Cl. tetani* is sited where steam penetration is likely to be poorest and where air layering or trapping is most likely to occur, is killed, there can be no doubt about the effectiveness of the process.

The primary division of sterility testing is between systematic control tests carried out by those responsible for the production of sterile products, and any other tests. Testing cannot be divorced from the technology of sterilising procedures without great loss of information, and subsequent examinations are to be regarded only as safeguards against the occurrence of gross contamination or complete process failure.

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THE TECHNIQUE OF STERILITY TESTING

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THE expanding use in medicine of drugs administered by parenteral routes has brought with it an increase in application of aseptic techniques and consequently in the development of reliable tests for sterility. Sterility tests were introduced in the British Pharmacopœia for the first time in 1932. Before this date the Regulations[1] made under the Therapeutic Substances Act, 1925, had specified such tests for certain vaccines, toxins, sera and similar products as well as for insulin and arsphenamines. From time to time other substances such as surgical ligatures, blood products and the antibiotics were added, and all were consolidated into the new Regulations[2] of 1952. The purpose of the prescribed tests is to detect the presence of living aerobic and anaerobic micro-organisms; specifications, but not formulæ, for suitable culture media are outlined in the Regulations and in the B.P.

The mode of all tests for sterility on preparations for injection is based on four principal premises; 1, that proper aseptic techniques have been carried out during processing, 2, that the procedure followed in making the test eliminates as far as possible the risk of introducing accidental contamination at this stage, 3, that the culture medium used is capable of detecting "small numbers of the commoner contaminating micro-organisms"[2], particularly pathogenic types or those which might cause spoilage of the medicament, and 4, that any bacteriostatic substance in the preparation is sufficiently diluted or neutralised to render it inactive.

On the first premise, it is clearly a prerequisite that before a preparation is submitted to a test for sterility it must have been subjected to such a treatment as can be reliably expected to yield a sterile product. The exclusive purpose of the test is to check that the approved process has been carried out satisfactorily; the test cannot of itself check that the process is satisfactory. On the second premise, it is recognised that an occasional growth could occur in the test which either originated from the injection or was introduced adventitiously during testing. To check this, the Regulations allow a second test, and, if necessary, a third test to be made, provided the organisms found in the first and second tests are manifestly different. The material is satisfactory only if one of the tests is completely free from growth. It follows, therefore, that the manipulative technique of testing should be as methodical as possible to minimise contamination from outside sources. A training scheme for operators in aseptic techniques was outlined by Coulthard[3], who also emphasised the importance of continuous bacteriological control of the premises, apparatus and methods employed for successful work of this nature.

TECHNIQUE OF STERILITY TESTING

Methods employed. In spite of the importance of sterility testing, comparatively little has been published on the actual techniques of carrying out the tests. Berry[4] drew attention to this deficiency and

Hopkins[5] has attempted to outline some of the precautions necessary. Whether one is required to carry out large numbers of tests regularly or only an occasional test, the principle and the precautions to be followed are essentially the same—the conditions under which the tests are made must be so designed and controlled as to eliminate outside contamination.

To this end the work should be carried out in a small "sterile" room —a room 6 ft. square is adequate—supplied with a flow of sterile-filtered air, the operations being conducted under an inoculating screen, also fitted to allow a controlled flow of sterile air. The room should contain only a necessary minimum of equipment so that it can be completely and easily disinfected. The sterile air supply must always be directed to carry contamination away from the site of operation. A regular "drill" of disinfecting the room and screen, and of assembling the test samples, sterile syringes and culture media must be followed, and the operators concerned should be dressed in sterilised gowns, headgear and rubber gloves and should disinfect their hands and arms with a non-irritating disinfectant, such as one of the proprietary chloroxylenol preparations. The tests must be made with a minimum of movement and air disturbance by the operators; each tube or bottle of medium must be "flamed" before opening and after inoculating, and the exteriors of the test ampoules or vials must be rendered free from contamination. This may be effected by immersing in 70 per cent. ethanol containing 1 per cent. of hydrochloric acid, and draining. Syringes are preferred to pipettes for inoculating the test media; they should be changed with each batch of material, and several times between batches, to avoid contamination. When ampoules are to be tested, their tops must be removed carefully with the aid of a small file; with vials the rubber cap should not be removed but samples should be withdrawn through the cap by means of a syringe. To facilitate this withdrawal, it is useful, before inserting the syringe into the cap, to draw into the syringe sufficient sterile air to replace the sample volume.

An improved testing method described by Royce and Sykes[6] dispenses with the majority of the preliminary preparations usually associated with aseptic manipulations and virtually eliminates the chances of introducing contaminations during testing. It uses a hermetically sealed screen and depends on the sterilising action of gaseous ethylene oxide. Obviously, access of the sterilising gas to the test sample or to the culture media must be excluded, hence the method can be used only when the samples are in containers such as ampoules, vials or bottles sealed from the air, and when the test media are also in screw-capped containers. The use of screw-capped containers in place of cotton wool plugged tubes for culture media was advocated some years ago[7], and to-day they are in common use, as for example in the well-known "McCartney bottles". The screen is fitted with long-sleeved rubber gloves fixed in such a position that the hands and arms are relatively free to carry out the normal operations within the screen. For use, it is loaded with the test samples, the sterile culture media, sterile syringes and all other necessary ancillary equipment. The volume of gaseous ethylene oxide introduced is 12·5 to 15 per cent. (v/v) and the screen is sealed and left for 18 to 24 hours during which

sterilisation of the whole of the exposed surfaces takes place. The ethylene oxide is removed by aspirating sterile-filtered air through the screen after which the inoculations of the test samples into the media are carried out normally. Provided adequate care has been taken in preparing the screen and its contents and in introducing the ethylene oxide, the danger of introducing accidental infection from outside sources is practically non-existent.

An entirely different method for making tests on liquid preparations is the filtration technique of Davies and Fishburn[8] designed primarily to overcome the carry-over of inhibitory concentrations of bacteriostatic substance into the test media. It has a number of commendable points, but the risk of contamination during manipulation cannot be discounted[9]. The procedure consists simply of passing the test sample aseptically through a small sterilised asbestos filter-pad and then culturing the whole of the pad in nutrient broth. The authors recommend using two pads, one for the aerobic test and one for the anaerobic test.

Testing Oils. The problem of freeing bacteria from an oily coating to allow them to proliferate in the surrounding nutrient medium is a difficult one, and for this reason the sensitivity of sterility tests on oils is much lower than on aqueous preparations. A number of media, including milk, gelatin and agar "shake" tubes, have been tried in the past with variable success. Davies and Fishburn[8] claimed that their filtration method is particularly suitable for this purpose and much more sensitive because the pads can be washed free from the oil by quickly rinsing through with light petroleum, thus removing the protective oily coating from any organisms present. Bullock and Booth[10] did not find any increased sensitivity by the filtration method and considered one of the most satisfactory procedures to be that of shaking the broth-oil mixture on several occasions during the incubation period. This is advocated in the B.P. 1953, and in the U.S.P. XIV[11].

Control Tests. Whatever testing system is employed, it is essential that the technique is continuously and adequately controlled. Timoney[12] mentioned the need for control tests on the media used, and Foord, Crane and Clark[13], discussing the testing of food containers, considered the hazards of aerial contamination to be so great that when examining for "absolute sterility" they felt it necessary to have at least one control for every three containers tested. Control infers proving that (*a*) the medium is sterile, (*b*) the syringes employed for inoculating the test samples are sterile, and (*c*) the testing procedure is carried out aseptically. The first of these is checked by incubating samples from each batch of medium at 37° C. and at about 25° C. for several days; the second can conveniently be checked by drawing broth into each syringe immediately before use, and injecting half into aerobic broth and half into anaerobic broth and incubating; the third is checked by making at intervals actual sterility tests under normal conditions, but with materials which have been sterilised in the laboratory by a reliable method. Water or saline sterilised in the autoclave in ampoules or vials is a convenient check material for liquid preparations, and sodium chloride sterilised in the hot-air oven is suitable for solid preparations.

Check tests of the foregoing type give valuable information on the reliability of the operational methods employed; as such they are indispensable features of a complete sterility testing system.

Temperature and Time of Incubation. After the sterility test media have been inoculated with the appropriate samples, they are incubated at 37° C. for five days. This temperature is chosen because it is the optimum for most types of micro-organism likely to be encountered, but there are many organisms capable of causing trouble which do not grow at this temperature. A "Memorandum on Avoidable Meningitis" issued with the authority of the Ministry of Health and reported in *The Pharmaceutical Journal*[14] discussed the causes of infection after spinal injections and found that "the organisms most frequently incriminated are *Ps. pyocyanea* and related organisms which can multiply in water at room temperature. . . . Some of the water bacteria fail to grow in ordinary media incubated at 37° C.". Wetterlow, Kay and Edsall[15] also commented on missed contaminations of psychrophilic organisms, that is, organisms incapable of growth at 35–37° C., due to incubation of tests at this temperature range. Such organisms are said to be found fairly frequently as contaminants in animal sera, blood, and other products of biological origin and in preparations containing no preservative; it is on record[16] that coliforms, which escaped detection at 37° C. but grew well at 23° C. have been recovered from human albumin. The obvious answer is to institute tests incubated at 20–25° C. as well as at 37° C. The U.S.P. follows this principle, albeit in a somewhat accidental and indirect manner, by including tests for moulds to be incubated at 25° C. A Sabouraud liquid medium is specified for this purpose, but it would certainly allow the growth of many psychrophils.

An incubation period of five days is required to allow organisms, present in only small numbers and possibly "damaged" by adverse bacteriostatic conditions, to grow and produce a visible turbidity. The Therapeutic Substances Regulations of 1927[1] specified seven days. Several investigators have commented on the long dormancy periods required for proliferation by some bacteria after a mild disinfectant or heat treatment; in particular, Burke, Sprague and Barnes[17] working with aerobic organisms including *Escherichia coli*, and Esty and Meyer[18] working with anaerobes observed delays in germination of periods of from several days up to a year or more. A useful review on this aspect of the subject was presented in 1930 by Morrison and Rettger[19], and later Wynne and Foster[20] described studies on the physiology of spore germination in *Clostridium botulinum* cultures.

It is desirable during the incubation period to examine the tests on several occasions, as it is not unknown for an organism to grow into a visible turbidity, and then settle leaving a clear supernatant broth which unless examined carefully, could give a false impression of sterility.

Culture Media Used in Sterility Testing

Criteria for Media. The purpose of a test for sterility is to ensure that the medicament is free from all contaminating micro-organisms. However desirable this may be in theory it cannot be achieved in practice

because of the wide, and sometimes specific, nutritional and temperature requirements of different bacterial and fungal types. Therefore, a compromise has to be made, and, to quote the Therapeutic Substances Regulations[2], "The tests . . . shall be made in a fluid medium, which, as sterilised for use, shall be capable of promoting the vigorous growth of the commoner contaminating micro-organisms, both aerobic and anaerobic". Miles[21] suggested that *Staphylococcus aureus* spp. should be included in the range of micro-organisms used for testing aerobic media, and *Clostridium œdematiens* for anaerobic media.

Not only is it a criterion that a satisfactory medium must support the growth of as wide a range of bacterial types as possible, but also that it shall support their growth when inoculated in small numbers. Such tests should be comparative with an approved medium and it is important to remember that the choice of the volume of medium in the container may be significant. Thus, growth from the same small inoculum may take place more readily in 10 ml. than in, say, 500 ml. of the same broth.

The most generally recommended media are those made with an extract or enzymic digest of meat. Such media, containing peptones and a range of amino-acids as well as other "growth factors" are sufficiently nutrient for most micro-organisms. However, the more fastidious streptococci and pneumococci might not grow readily in them, and certainly tubercle bacilli would not be detected within the limited five-day incubation period; neither could the viruses proliferate. But, it is reasonable to assume that such organisms could not be introduced as contaminants in pure culture. They would almost certainly be in association with the commoner staphylococci or Gram-negative organisms, all of which should grow readily in the normal media.

Choice of Media. The B.P. 1953, states that the medium used for aerobic organisms "either consists of meat extract containing a suitable concentration of peptone or is prepared by the enzymic digestion of protein material", and that for anaerobic organisms is similar but with the addition of either heat-coagulated muscle or about 0·05 per cent. of agar with a substance to reduce the oxidation-reduction potential of the medium. One of the first digest media, as distinct from simple meat infusions, was that described by Douglas[22], made by a short period tryptic digestion at 45° C. of bullock heart. Later, Hartley[23] modified the process by using an overnight digestion at 37° C. of lean horse flesh. Subsequently, several further modifications have been suggested, mainly in relation to the source and quantity of meat. A digest of bullock heart using half the quantity of meat of the Hartley formula has been found sufficiently nutritive to allow the growth of the pyogenic streptococci[24]. Barnes[25] has shown recently that the method of extracting the enzyme from pancreas determines to a large extent the nutritive properties of a tryptic digest broth. Alcoholic extracts yielded more satisfactory broths than did acid-extracted or commercial trypsins. She considered the difference to be due not to growth factors provided by the alcoholic extract but to supplementary non-proteolytic enzymes, e.g., amylase and lipase, which would not be present in the acid extract. Papain can be used in the

place of trypsin[26]. In this case the digestion is usually carried out at a higher temperature for a shorter period—60° C. for 4 to 6 hours is commonly employed. It gives a more complete digestion so that a smaller amount of meat can be used to produce the same volume of broth. In Table I, the nutrient properties of typical trypsin and papain digest broths are compared in terms of the rate of growth of small inocula of *Staph. aureus* in 10 ml. of medium at 37° C. Both types of media give a relatively short lag-phase of some two hours, after which the organisms grow with normal rapidity. By way of contrast, an unsatisfactory trypsin medium is included.

Because of possible variations in the source and quality of the meat used and also in the rate and type of digestion, it is convenient to standardise digest media by their amino-acids content and biuret reaction. But this alone does not assess the nutritive properties of the medium. Many other growth factors occurring naturally in meat must also be present.

TABLE I

RATE OF GROWTH OF *Staph. aureus* IN MEAT DIGEST BROTHS

Period of incubation of inoculated broth (hrs.)	Viable bacteria per ml. in :—				
	Trypsin broth A	Trypsin broth B	Papain broth A	Papain broth B	Trypsin broth (unsatisfactory)
Initial inoculum	6	3	6	7	6
1½	6	8	8	23	4
2	7	9	16	49	7
2½	22	11	40	136	1
3	61	51	86	310	1
3½	183	106	225	c 1000	2
4	500	223	500	>1000	1
4½	c 1000	500	c 1000	>1000	0

All tubes were incubated at 37° C.

For this reason the quantity of meat used in a digestion must be standardised and must not be reduced too far, otherwise the resultant broth will be deficient in these growth factors. This is well illustrated in some experiments made on a papain digest of the residues of meat after trypsin digestion. Both the trypsin and the papain broth gave satisfactory amino-acid titration values, but the papain digest, being made from exhausted meat tissues, was deficient in soluble growth factors and consequently did not adequately support the growth of staphylococci.

Arising from anticipated war-time shortages of meat, Gladstone and Fildes[27] devised a medium without peptone or meat extract. It is made with acid-hydrolysed and tryptic-digest caseins, yeast extract, sodium glycerophosphate, sodium lactate, and a small amount of glutamine. It is claimed to support the growth of organisms met with in medical bacteriology even better than do meat media, and to be cheap to make. Brewer[28] also suggested a meatless medium made by the papain digestion of vegetable meals such as those of cotton seed, peanut, soya-bean, whole and sprouted grains and other seeds. He found it equal to, or more satisfactory than, meat media for general culture purposes, but it does not seem to have been adopted for normal use.

In preparing media for routine use, it is important to check each batch for its ability to support growth, because sometimes inhibitory contaminating substances may inadvertently gain access during processing. Attention was first drawn to this by Dubos[29], who observed the phenomenon in certain commercial peptones. He was not aware of the nature of the substance, but found it to cause bacteriostasis only under oxidising conditions and not at all under reducing conditions. Later O'Meara and Macsween[30] investigated the reasons for the failure of staphylococci to grow in certain meat digest and peptone media. They attributed it to copper dissolved from the metal pans during the digestion process. Marked inhibition was exhibited by as little as 4 p.p.m. of copper, a concentration "of the order commonly found in routine media", but the effect was not direct. It could only be demonstrated by heating the medium and then keeping it for some time; and it was abolished by reheating. Examining the action further, Woiwod[31] attributed it to the formation in the medium of colloidal copper sulphide during the heating process. The mechanism of the action is enigmatical, since no inhibition occurs in media heated in sealed containers, and it is confined to staphylococci and other Gram-positive organisms. Colloidal sulphur and the sulphides of iron and manganese are also inhibitory but to a much less extent. In this connection, a recent report on the chemical analyses of different peptones[32] is of interest.

In 1929, Dubos[33] observed losses in the nutritive power of media after keeping them for several weeks. On the basis that all micro-organisms grow better under somewhat reduced oxidation-reduction potential systems, he was able to show that it could be restored by reducing the oxidation-reduction potential of the system by heating, by reducing with hydrogen or by adding cysteine or blood. In a later publication[29] he showed that reduced thiol compounds were always effective and so advocated the addition of thioglycollic acid, or its sodium salt.

Other investigators have reiterated the value of thioglycollate broth. Thus, Reid and Bowditch[34] believed it to be superior to ordinary broth or blood media for diagnostic purposes, and Marshall, Gunnison and Luxen[35], Fay and Blubaugh[36], Graydon and Biggs [37] and Berry[4] found it more effective in sterility testing, particularly with biological materials containing a mercury compound as the bacteriostatic agent. Brewer[38] also found it advantageous in a medium containing "pork infusion solids", peptone and a small amount of agar for detecting and cultivating anaerobic organisms. The U.S.P. adopted a modification of the so-called "Brewer's medium" for use in sterility testing, the modified medium containing L-cysteine, yeast extract, casein digest, glucose and sodium thioglycollate, with agar as an optional addition to give a semi-solid fluid. The B.P., 1953, now recommends a similar medium. The obvious advantages of a thioglycollate medium are that it is clear for ease of reading, it detects aerobic and anaerobic organisms simultaneously, and it neutralises the action of certain bacteriostatic agents. Care must be taken, however, to use freshly made medium, as it can become inhibitory due to deterioration on storage[39,40]

The inclusion of agar to increase the viscosity of the medium was first advocated by Hitchens[41] in 1921, who claimed that a 0·1 per cent. concentration gave greater sensitivity in the detection of both aerobic and anaerobic organisms. This was confirmed later by Spray[42] working with anaerobic organisms alone. Falk, Bucca and Simmons[43] investigated the phenomenon more closely using the hay bacillus, staphylococci, streptococci, Gram-negative organisms and diphtheroids, and found the optimum concentration of agar to range between about 0·06 and 0·25 per cent. This was most marked when the inocula of organisms were small, that is of the order of 10 per ml. and a concentration of 0·1 per cent. was finally recommended.

Media for Anaerobic Organisms. For the cultivation of anaerobic organisms any ordinary nutrient medium may be used at a low oxidation-reduction potential. Satisfactory reducing conditions can be attained in practice by excluding aerial oxygen with a soft paraffin or other seal, or by adding to the medium reducing compounds such as alkaline sulphides, pyruvic acid, ascorbic acid, cysteine, glutathione or thioglycollic acid. As far back as 1917, Douglas, Fleming and Colebrook[44] suggested putting rusty nails in ordinary culture media to encourage the "aerobic" growth of *Clostridium butyricum* and *Cl. œdematiens*, and much later Hayward and Miles[40] took this further by suggesting the use of strips of sterilised mild steel dropped into tubes of media as required. The iron did not adversely affect the nutritive properties of the media, which were considered superior to thioglycollate media in that the latter tend to deteriorate on storage. The idea was put forward in the first place in lieu of the McIntosh and Fildes jar as a means of studying anaerobic bacteria, but it might easily be adapted for sterility testing. The principal disadvantage is that the iron acts purely in a reducing capacity and has no power to inactivate bacteriostatic agents, whereas other reducing agents, such as meat and thioglycollate, act in the dual capacity. *En passant*, a specific test used by Pulvertaft[45] for detecting tetanus spores in sanitary towels was to place each towel in a sterile Kilner jar, cover it with boiling broth, immediately screw down the lid and incubate at 37° C. Such a test obviously could only be used for detecting heat-resistant spores.

The classical medium employed for many years in sterility testing for anaerobic organisms is based on Robertson's meat medium, that is, a nutrient broth with a deep layer of minced, lean meat at the bottom; any meat can be used, but heart muscle is preferred to flesh[46]. The meat is prepared by pre-cooking in a weak alkali solution; raw meat can be used but it tends to give an opalescent and "stringy" appearance to the final medium. Such a medium when freshly sterilised is sufficiently oxygen-free to allow the growth of obligate anaerobes, but it fairly quickly re-dissolves oxygen on standing. Marchal[47] observed that, for maximum sensitivity in detecting small numbers of anaerobic bacteria, not only is it desirable to have meat present, but also the medium should be covered with a liquid paraffin seal. It is, therefore, recommended that for sterility testing meat media should be sealed with a layer of soft paraffin and be heated and cooled immediately before use. The function of the

seal is to reduce the rate of dissolution of oxygen and the heating is to drive off any oxygen which might have gained access. The relative capacities of meat media with and without a seal to support the growth of two species of *Clostridium*, *Cl. welchii* and *Cl. sporogenes*, are illustrated in Table II.

TABLE II

GROWTH OF ANAEROBIC ORGANISMS IN MEAT AND THIOGLYCOLLATE MEDIA

	Growth* of *Cl. welchii* in:—				Growth* of *Cl. sporogenes* in:—			
Dilution of culture inoculated (1 in −)	Meat medium without seal	Meat medium with seal	U.S.P. thioglyc. medium	Meat medium +0·1 per cent. thioglyc.	Meat medium without seal	Meat medium with seal	U.S.P. thioglyc. medium	Meat medium +0·1 per cent. thioglyc.
1×10^{7}	+	+++	+++	+++	0	+++	+++	+++
3×10^{7}		++	+++	+++		+++	+++	+++
1×10^{8}	0	+	+++	+	0	0	+++	+++
3×10^{8}		+	+	++		+	+	+++
1×10^{9}	+	0	0	+	0	0	0	+
3×10^{9}		0	0	0		0	0	0
1×10^{10}	0	0	0	0	0	0	+	+

* 50 ml. tubes of medium used, and inoculated with 1 ml. of diluted culture;
3 tubes inoculated at each test level; +++, ++, + and 0 = no. tubes showing growth.

The function of the meat in the medium is somewhat obscure. Lepper and Martin[46] suggested that it carries natural reducing systems in the form of glutathione and thiol groups and of unsaturated fatty acids, which are catalytically oxidised by the hæmatin present in the denatured muscle protein. Knight[48] suggested in addition that the meat fibres provide a nidus to assist in initiating proliferation of the organism. Evidence to support the latter point had earlier been provided by Marchal's observation[47] that asbestos could equally well take the place of meat in the medium.

According to Knight[48], a meat medium has only a small reducing capacity which is gradually and irretrievably lost on storage due to the slow natural absorption of oxygen. It is, therefore, desirable to add suitable agents to increase the reducing capacity. Of the several agents possible, thioglycollic acid seems to be the most suitable. It had been suggested as early as 1926 by Quastel and Stephenson[49] as a means of cultivating anaerobes; Fildes[50] used it in his studies of anaerobic bacteria, and McClung[51] advocated its inclusion in infusion media for the large scale cultivation of anaerobes. As stated earlier, Brewer[38] independently devised a thioglycollate medium for the "aerobic" cultivation of anaerobes, and such media are frequently referred to by his name. The relative growth capacities of thioglycollate and of meat media are illustrated in Table II. Thioglycollate media have been commented upon favourably by many other workers e.g.[4,29,33,38]. They have the advantage that they do not require a seal, they do not need to be re-heated prior to use, and they retain their anaerobic properties for long periods. Moreover, they can be used facultatively because they allow the growth of micro-aerophilic and aerobic organisms, and the thioglycollic acid combines with and inactivates some bacteriostatic agents.

Media for Moulds and Yeasts. As well as tests for organisms growing at 37° C., the U.S.P. requires tests to be made for the presence of moulds and yeasts by incubating inoculated media at 22° to 25° C. for at least 14 days. The British regulations do not require such tests, but they are commonly applied, particularly to injections known to be subject to contamination by mould, yeast or any bacteria with optimum growth temperatures below 37° C.

The medium specified in the U.S.P. XIII was a honey medium made with enzyme-digested casein and 6 per cent. of honey, but in the next revision it was changed to a modified Sabouraud liquid medium containing peptone and 2 per cent. of dextrose, adjusted to pH 5·7. Several other media could be used satisfactorily, such as wort agar or malt agar or plain glucose agar or glucose broth. The primary requirements are that the medium should contain carbohydrate, that the pH value should be on the acid side, that is, ranging between pH 5 and pH 6, and that the incubation temperature should be at about 25° C.

Inactivating Agents

Many of the medicaments administered by injection to-day are themselves antibacterial, and their numbers are increasing as a result of the introduction of the newer antibiotics and chemotherapeutic agents. Other injections, particularly those dispensed in multidose containers, contain an added bacteriostatic agent. Those most generally added to injections are phenol, cresol, chlorocresol, chlorbutol, benzyl alcohol, esters of *p*-hydroxybenzoic acid, quaternary ammonium compounds and compounds of mercury such as phenylmercuric nitrate and thiomersal. Unless due care is taken the antibacterial properties of the preparation can be carried over in the sterility test, resulting in false-negative readings being obtained from samples which are actually contaminated. To meet this, the Therapeutic Substances Regulations[2] and the U.S.P. XIV[11] specify that when a bactericidal or bacteriostatic agent is present either it shall be diluted in the test with such a volume of medium as will render the agent ineffective, or it shall be treated with a suitable inactivating agent.

Inactivation by Dilution. The phenolic substances can all be dealt with by a simple dilution in the sterility test medium. The earlier Regulations[1] required their final concentrations to be less than 0·01 per cent., and, since their concentrations in the injections do not exceed 0·5 per cent., this was readily achieved by inoculating each 1 ml. of the test sample into 50 ml. of medium. It has been suggested[39] that a 0·5 per cent. solution of phenol or chlorbutol even when diluted fifty times is still capable of inhibiting germination of spores, and Flett and others[52] have produced evidence to show that *Staph. aureus* and *Salmonella typhi* can be revived after treatment with dilutions of phenol hitherto considered lethal (e.g., 1 in 65 said to kill in 10 minutes) by adding activated charcoal or ferric chloride to the recovery medium. There seems to be little support, however, for either of these views, in particular, neither Tilley[53] nor Jacobs and Harris[54] were able to confirm the latter.

As well as the antibiotics and sulphonamides, a number of other substances used in injections possess antibacterial properties. Thus, Davies and Fishburn[8] recorded "bacteriostatic concentrations" of hexobarbitone and phenobarbitone at a dilution of 1 in 10^3, thiopentone at 1 in 10^3 and mepacrine methanesulphonate at 1 in 10^4; Booth[5] advised diluting all barbiturates to a final concentration of less than 0·2 per cent. in the test broth and also drew attention to the need for reducing the pH value of some of the broth dilutions of the sodium salts which may be as high as pH 9. Kohli and others[56] and Gupta[57] also drew attention to the bacteriostatic properties of a number of drugs commonly administered by injection, and recommended suitable dilutions to be employed in testing. Adequate dilution will overcome the inhibitive properties of these compounds, but it is an essential part of sterility testing to ensure that the dilution has been sufficient by inoculating control tubes with small numbers of test organisms and observing their growth on incubation.

Inactivation of Mercurial Preservatives. Unlike the phenolic compounds, the compounds of mercury used as bacteriostatics are not inactivated by simple dilution in nutrient broth. They are adsorbed on the bacterial surfaces and can only be satisfactorily neutralised by chemical action. It has been suggested that adding charcoal to the medium is effective[58], but sulphydryl compounds have proved more acceptable. Cysteine has been used[59,60] but again, thioglycollic acid is generally preferred[39]. A broth containing 0·05 per cent. of thioglycollate is said to give more satisfactory results in testing sera containing thiomersal[37], and it is reported to give a greater number of positive responses from vaccines inoculated with staphylococci[36].

The value of thioglycollic acid in sterility test media has already been discussed. In the particular case of the mercurials, it would seem to act in two ways: (*a*) by neutralising the mercurial by chemical combination, and (*b*) by encouraging the growth of bacteria as a result of inducing a more favourable oxidation-reduction potential.

Inactivation of Arsphenamines. The various arsphenamines administered by injection all become highly toxic on exposure of their solutions to the air, such that dilutions up to 1 in 100,000 may be lethal or inhibitory to the commoner bacteria—Berry and Jensen[61] have put the figure even higher in some cases, finding the Gram-positive bacteria more sensitive than the Gram-negative. Ascorbic acid in the proportion of one part to three parts of neoarsphenamine or Mapharsan is reported to delay oxidation for at least forty-eight hours[62]. Minced meat was also found to mitigate the toxicity developed during testing, presumably because of the thiol compounds naturally present, and this was used for many years in the author's laboratory. However, a more sensitive and reliable medium was sought, and finally a meat and thioglycollate medium was chosen, on account of its efficacy in supporting the growth of small bacterial inocula, its stability and availability. The medium employed was a tryptic digest broth containing 0·4 per cent. of sodium thioglycollate and a layer of minced meat[63]. Meat was included because it gave slightly more favourable conditions for *Staph. aureus* and *Bacillus subtilis*. Berry

and Jensen[61] also concluded that thioglycollic acid or dimercaprol could be used satisfactorily. They also made the novel suggestion that where it is difficult to tell whether there is growth or not the differentiation might be made by observing changes in pH value.

Inactivation of Sulphonamides. The sulphonamides are frequently applied topically to open wounds; hence it is essential that they, as well as other powders thus applied, shall be free from contaminating bacteria, especially pathogenic anaerobes, and that adequate tests be applied to confirm it. There are a number of cases on record of deaths from tetanus following the application of non-sterile powders[64–67].

The antibacterial activities of the sulphonamides are known to be influenced considerably by other substances present in solution. Thus, *p*-aminobenzoic acid, methionine, purines, casein, albumin, meat extract, blood and serum all reduce their activities to a greater or lesser extent. Much has been written on the influence of culture media constituents and on the effect of different sized inocula of bacteria in relation to their resistance and growth in the presence of sulphonamides[67–72], all of which is, in effect, an expansion of Fleming's findings[73] that small inocula are more easily inhibited than larger ones and that adding peptone to media tends to inhibit bacteriostasis. On the basis that minced meat contains natural sulphonamide inhibitors, it has been used successfully by the author in routine sterility testing. Liver infusion media are reported to be of little value in inactivating sulphathiazole, and human serum to be more effective than human plasma, rabbit blood or horse serum[74]. The sulphonamide antagonists naturally occurring in most media are apparently neutralised by horse red blood cells but not by those of other animals[75].

Since the now classical work of Woods[76] in discovering the antagonising effect of *p*-aminobenzoic acid on sulphonamides, the most reasonable procedure seems to be to add a measured amount of the acid to culture media as required. It has been claimed[77] that Brewer's medium contains sufficient sulphonamide inhibitor to inactivate 50 mg. of sulphanilamide per 20 ml. of medium, but Brewer himself, as quoted by Long[78], preferred to add *p*-aminobenzoic acid. The amount required is small, since one molecule of the acid is said to be sufficient to antagonise 5000 molecules of sulphanilamide[76]. In practice, 250 ml. of a 0·01 per cent. solution of *p*-aminobenzoic acid in broth is sufficient to neutralise 5 g. of sulphanilamide or sulphathiazole.

Inactivation of Penicillin. Although solutions of the penicillin salts are labile, their stabilities over a few days are sufficient to upset any tests for sterility which may be applied to the substances without inactivation. For a successful test for sterility, the antibacterial properties should be destroyed as quickly as possible, certainly within an hour or two.

Penicillin is selectively antibacterial against Gram-positive organisms, and, in 1940, Abraham and Chain[79] prepared an extract of *E. coli* and of other Gram-negative organisms containing an enzyme, since called penicillinase, which was the responsible inactivating agent. The commercial enzymes, "taka-diastase" and "clarase", have been described as

penicillin inactivators and their use advocated in sterility tests for penicillin[80], but the active agent in these preparations is probably the same as the bacterial penicillinases.

It is important to use highly active preparations of penicillinase to reduce the time of inactivation of the penicillin, and one of the most satisfactory methods of preparation is to add penicillinase solution continuously by growing cultures of a strain of *B. subtilis* as described by Duthie[81]. Stability of the enzyme is of equal importance. Duthie claimed stability to heat at 100° C. for 20 minutes for this preparation, and Smith and Smith[82] described the preparation of an enzyme concentrate from a paracolon bacillus which was thermostable when dry, but completely unstable in solution. Manson and Pollock[83] and Manson, Pollock and Tridgell[84] have discussed the heat stabilities and other properties of penicillinase preparations from *B. subtilis* and *B. cereus*, finding considerable variations according to the source and method of preparation.

The United States Federal Register[85] prescribed hydroxylamine hydrochloride as the inactivator, used in a 1 in 300 solution. This is not a happy choice, however, and is to be discouraged, because of the known bacteriostatic properties of hydroxylamine. Even a 0·001 per cent. solution is said to be inhibitive[86]. Certain sulphydryl compounds can be used, but they are only effective in high concentration or when they are present in excess.

Inactivation of Streptomycin. Streptomycin and its salts are much more stable than is penicillin. It is claimed that cysteine[87], cevitamic acid[88], and carbonyl reagents[89] are effective inactivators. It has been shown, however[88], that several so-called "inactivating" agents, such as glucose, ketones and sulphydryl compounds may only be partially effective, and that some only reduce the activity of the streptomycin, which has optimum activity in slightly alkaline conditions, by rendering the medium acid. Cysteine is the most reliable of the inactivators suggested, its action being attributed to the blocking of an active grouping in the streptomycin molecule. Lipositol is said to inhibit streptomycin activity[90], but this has since been contradicted[91].

An interesting recent discovery is the substance produced by *Pseudomonas pyocyanea* first described by Sureau and others[92] and thought to be an enzyme. The substance has since been purified and crystallised by Bergman and others[93] and by Lightbown[94], and reported by the latter to be a mixture of several closely related derivatives of 4-hydroxyquinoline-*N*-oxide. It was claimed to have the property of inactivating streptomycin and also dihydrostreptomycin[91] and neomycin[93], and as such held out promise as suitable for use in sterility tests on these activities. Subsequent investigation has not fulfilled this promise[94], because the material itself is bacteriostatic and it only slightly antagonises the action of streptomycin against *E. coli*.

Inactivation of other Antibiotics. Apart from a suggestion of an inactivator for neomycin[93], there is no known antagonist against other antibiotics. In carrying out sterility tests, therefore, it is possible only

to use a reasonable dilution in broth of the sample. This means that an enormous volume of medium is required to test a reasonable amount of sample, or, the amount of sample tested is undesirably small. The tendency, as indicated in the B.P. test for sterility on aureomycin, is to accept the idea of using only a small sample. This cannot, however, be considered satisfactory.

Conclusions

The importance of carrying out adequate tests for sterility on preparations intended for injection or for topical application to open wounds cannot be over-estimated. Because of the anticipated low incidence of bacterial infection in the preparations, such tests must be made in media known to be capable of supporting the growth of small numbers of a variety of commonly occurring micro-organisms. They must include tests for aerobic and anaerobic bacteria growing at 37° C., and should also be capable of detecting moulds and psychrophilic bacteria; this can be done by setting up additional tests incubated at 22 to 25° C.

As well as checks on the nutritive properties of batches of media, control tests should also be made at intervals on the method of carrying out the tests, to ensure that the introduction of accidental contaminations is at a minimum.

Media used for aerobic tests can be made with peptone and meat extract or from the tryptic or papaic digestion of meat. Other sources of nutrients can be used, but confirmation of their ability to support growth of bacteria is essential. For anaerobic tests, nutrient broth with a layer of minced meat in the bottom and sealed with soft paraffin is satisfactory, but other reducing agents, in particular thioglycollic acid, have advantages over meat.

Adequate precautions must be taken to render any bacteriostatic substance in the injection inactive. In the case of phenolic substances simple dilution in the test broth is sufficient, but mercurial bacteriostatics require a more positive inactivator. Thioglycollic acid is the most satisfactory. In some cases the medicament is itself bacteriostatic or bactericidal, but this can generally be overcome by dilution in broth. The antibiotics, however, need special treatment. Penicillin can readily be inactivated by using a potent penicillinase preparation; streptomycin can be inactivated effectively by using cysteine in the medium. No inactivator is known for other antibiotics, and the position with them is, therefore, less satisfactory.

I wish to express thanks to several of my colleagues who have helped to contribute much of the information contained in this paper, especially to Mr. C. E. Coulthard under whose guidance I first learned the art of sterility testing.

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RESEARCH PAPERS

THE ANTICOAGULANT ACTIVITY OF DEXTRAN SULPHATE

II. THE EFFECT OF DEXTRAN SULPHATE ON THE ONE-STAGE PROTHROMBIN TIME

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IN a previous paper[1], the anticoagulant activity of dextran sulphate was compared with that of heparin. The experiments were designed to study the effects of the two drugs on various stages of the clotting sequence. This communication deals with the effects of the drugs on one simple and widely used clotting test, the one-stage prothrombin time[2,3].

As Biggs and Douglas[4] have pointed out, the so-called prothrombin time with brain thromboplastin does not necessarily measure prothrombin. In this test, the clotting time is prolonged by a reduction in clotting factors V, VII or, to a lesser degree prothrombin[4]; and also by a reduction in fibrinogen concentration lower than about 0·05–0·10 g. per cent[5]. There is evidence that the clotting time is shortened by an abnormal increase in factor V activity in certain cases of thrombosis[6] and by the increased titre of factor VII which occurs in pregnancy[7]. It is probable that the test proceeds in three stages: first, the brain reagent is activated by factor V and factor VII[8,9]; then, prothrombin is converted to thrombin; and finally, fibrinogen is converted to fibrin. Clearly, a lengthening or shortening of the prothrombin time can be caused by acceleration or delay in any one or more of these stages.

From the previous work[1] it may be inferred that, by comparison with heparin, dextran sulphate interferes but feebly with the reactions of the second and third stages of the prothrombin time test.

EXPERIMENTAL

Materials

Citrated plasma was obtained by centrifuging a mixture of 9 parts of normal venous blood and 1 part of 3·8 per cent. (w/v) trisodium citrate (anhyd.).

Human brain thromboplastin, acetone-dried extract[10].

Russell's Viper venom ("Stypven"), 1 : 10,000 reagent solution.

Dextran sulphate and heparin. As in the previous paper[1], in which details of the various preparations were given, the drug concentrations are expressed in terms of International Standard heparin (130 u./mg.) and British Standard dextran sulphate (on the basis of 25 heparin u./mg.).

Special reagents are described in connection with the experiments in which they were used.

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† In receipt of a grant from the Medical Research Council.

Methods

The performance of the prothrombin time test followed the usual principle[11], in which 0·1 ml. of citrated plasma received 0·1 ml. of brain or viper venom and 0·1 ml. of M/40 calcium chloride solution. It was sometimes convenient to prepare the brain or the viper venom in M/40 calcium chloride solution, so that the two reagents were added together in 0·1 ml. The final volume was usually restricted to the conventional 0·3 ml., although in some cases it was increased to 0·4 ml. but the general form of the test was not altered. The observations were made in a water bath at 37° C. As in the previous work, replicate readings, usually 4, were made in a balanced random order to eliminate systematic errors related to the passage of time[12].

Where data are brought together from different experiments with differing control clotting times, the results are presented as the ratios of the test clotting times to the corresponding control clotting times. On the graphs, the control times are thus represented by horizontal lines at unity. The actual control clotting times (sec.) are then also given in the legends.

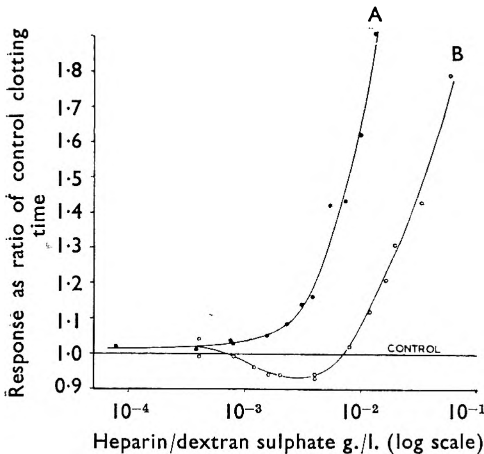

FIG. 1. *The accelerating effect of low concentrations of dextran sulphate.* (Experiment 1.) Each point represents the mean of 3–5 replicate readings of one-stage prothrombin time, expressed as a ratio of the mean control time obtained in the same experiment. Range of readings with 0·85 per cent. (w/v) sodium chloride solution ("saline control") in the six experiments represented, 19·9–22·6 sec. Commercial, clinical solution of heparin; Injection of Dextran Sulphate. A, heparin; B, dextran sulphate.

Experiment 1: *The accelerating effect of low concentrations of dextran sulphate.* Figure 1 shows the effect on the prothrombin time of varying concentrations of heparin or dextran sulphate; the other constituents of the test, and the final volume, were unaltered.

The heparin curve is much as might be expected, but the dextran sulphate graph shows that the drug causes a definite shortening of the clotting time at concentrations just less than those at which a lengthening is produced. This effect has varied in magnitude between 5 and 20 per cent. of the control time; and also, the range of concentrations over which it has been observed has varied from the 10-fold range of the Figure to about 100-fold, but with the shortest clotting times always occurring between 10^{-2} and 10^{-3} g./l. of dextran sulphate. Nevertheless, the effect has been clearly evident each time it has been sought.

The present paper is concerned with the investigation of this accelerating effect.

1. *Effect of varying the plasma and brain reagents*

i. *Experiment 2: Variation in brain reagent concentration.* Prothrombin times were obtained in the presence of 5 concentrations of dextran sulphate, using two concentrations of brain suspension, with the same concentration of plasma throughout. The results are illustrated in Figure 2, which shows that the acceleration was more marked with the higher concentration of brain.

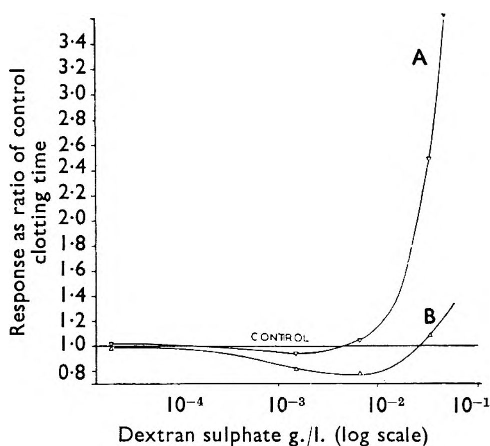

FIG. 2. *Variation in the concentration of the brain reagent.* (Experiment 2.) Each point represents the mean of 4 replicate readings of one-stage prothrombin time, expressed as a ratio of the mean control time with the same concentration of brain; the mean saline control readings were: with concentrated brain, A, 25·2 sec.; with brain diluted 1 : 50, B, 36·5 sec. British Standard dextran sulphate.

ii. *Experiment 3: Variations in plasma concentration.* Prothrombin times were obtained with 5 concentrations of dextran sulphate using three concentrations of plasma and the same concentration of brain suspension throughout. The results (Fig. 3) showed that the degree of acceleration varied directly with the plasma concentration, over the tested range.

2. *Effect of the addition of heparin*

Experiment 4. Three concentrations of dextran sulphate were chosen so that the highest and lowest gave clotting times immaterially different from the control time, but in the presence of the intermediate concentration the clotting time was definitely shortened. Ten replicate readings were obtained of the control time alone, and of the clotting time with each of these concentrations of dextran sulphate both with and without the addition of a concentration of heparin sufficient to prolong the control clotting time to twice its original value. The mean results are given in Table I, and show that the proportion by which the clotting time is shortened by $1{\cdot}6 \times 10^{-3}$ g./l. of dextran sulphate is not varied by the anticoagulant effect of $6{\cdot}4 \times 10^{-3}$ g./l. of heparin. This suggests that the accelerating effect of dextran sulphate and the anticoagulant effect of heparin are not mutually exclusive.

3. *The Site of Action of the Accelerating Effect*

When the accelerating effect was noted in the prothrombin time test, attempts were made to detect an acceleration in the clotting time of whole blood. Tests were made both in glass tubes and silicone-coated tubes, but no suggestion of an acceleration was observed. Thrombin generation tests[13] were also made, with various concentrations of dextran sulphate less

TABLE I

COMBINED EFFECT OF ANTICOAGULANT CONCENTRATION OF HEPARIN WITH ACCELERATING CONCENTRATION OF DEXTRAN SULPHATE

Heparin concentration, g./l.	Dextran sulphate concentration, g./l.			
	0	$2 \cdot 0 \times 10^{-8}$	$1 \cdot 6 \times 10^{-3}$	$2 \cdot 3 \times 10^{-2}$
0	19·3 sec.	20·1 (+4 per cent.)	15·4 (−20 per cent.)	19·3 (±0 per cent.)
$6 \cdot 4 \times 10^{-3}$	39·8	39·6 (−1 per cent.)	32·7 (−18 per cent.)	41·0 (+3 per cent.)

Each entry is the mean prothrombin time in seconds derived from 10 replicate readings. The percentages in parentheses show the amounts by which the entries differed from the corresponding control clotting times obtained without dextran sulphate (Col. 2).
International Standard heparin: British Standard dextran sulphate.

than those used in anticoagulant tests reported in the previous paper[1], but using a similar method to eliminate bias related to order of testing, and again, acceleration was not observed. This was taken to mean that the affected reaction either did not occur in the spontaneous coagulation of blood or, that if it did, it occupied a very small proportion of the interval elapsing before thrombin appeared in the system.

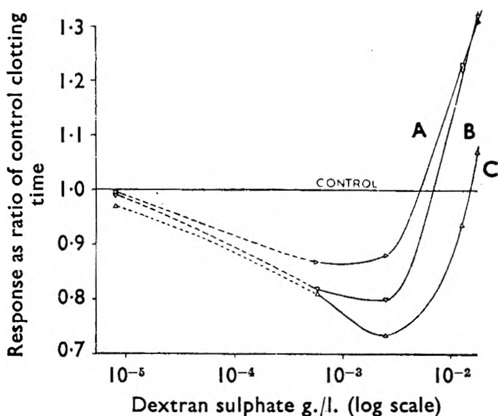

FIG. 3. *Variations in the concentration of plasma.* (Experiment 3.) Each point represents the mean of 3 replicate readings of one-stage prothrombin time, expressed as a ratio of the mean saline control time with the same concentration of plasma; the mean saline control readings were: 38 per cent. plasma C, 28·3 sec.; 19 per cent. plasma, B, 29·1 sec.; 9·5 per cent. plasma, A, 35·0 sec. In this experiment, 0·05 ml. of dextran sulphate solution was added to 0·15 ml. of plasma or diluted plasma; to this was added 0·1 ml. of brain suspension and 0·1 ml. of calcium chloride. The calcium chloride solutions were adjusted as follows: for 38 per cent. plasma, M/27; for 19 per cent. plasma, M/54; and for 9·5 per cent. plasma, M/108. British Standard dextran sulphate.

In the previous paper[1] evidence is given that dextran sulphate has no effect upon prothrombin conversion, and only a mild inhibitory effect upon the thrombin fibrinogen reaction. This suggests that in the prothrombin time test, the acceleration operates upon the reaction(s) between the brain reagent and factors V and VII[8,9]. To test this hypothesis the following experiments were made.

i. Experiment 5: *Experiment 1 repeated with various sources of "thromboplastin".* Prothrombin times were obtained with a single concentration of plasma, a range of concentrations of dextran sulphate, and with, in turn, brain extract, Russell's Viper venom and blood thromboplastin[14]. The results are given in Figure 4, which shows that the acceleration was equally well shown by brain and venom but was absent with blood thromboplastin.

ii. *Experiment 6*: *"Prothrombin time" with brain and purified clotting factors.* (*a*) A similar experiment was made by adding brain extract and calcium chloride to a mixture of purified clotting factors and a series of concentrations of dextran sulphate. The mixture contained fibrinogen (Lister Institute), the dialysed eluate from alumina[14] which had been incubated with normal plasma (as a source of prothrombin, the factor VII content being ignored[10]), the precipitate thrown down between 33 and 50 per cent. saturation of normal alumina-plasma with ammonium sulphate (as a source of factor V[10]) and normal serum (as a source of factor VII[10]), buffered with 0·0017M amino*tris*-(hydroxymethyl)methane[15] at pH 7·30. This confirmed (Fig. 5, *Direct* curve) that the acceleration occurred equally well in this mixture.

(*b*) The serum and factor V were then mixed with the brain and, after 3 min. incubation, sub-samples from this mixture were added to the remaining components. In five experiments, testing altogether a 1000-fold range of dextran sulphate concentrations, there was no evidence of an acceleration. Each experiment yielded duplicate readings against 5–8 drug concentrations, obtained in a balanced order to eliminate bias arising from instability of the activated brain. The mean results are shown in Figure 5, *Pre-incubated* curve.

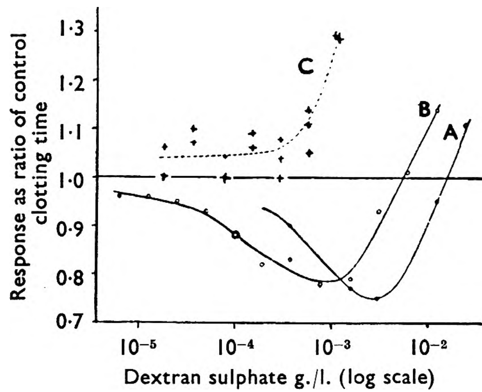

FIG. 4. *Different thromboplastic reagents.* (Experiment 5.) A: Brain suspension. Collected results from two series of tests: thus, each point represents mean of 2–6 readings of one-stage prothrombin time. Mean saline control clotting time, 25 sec.

B: Russell's Viper venom. Each point represents the mean of three replicate readings of one-stage prothrombin time. Mean saline control clotting time, 32 sec.

C: Blood Thromboplastin[14], prepared by incubating together alumina-plasma, serum, antihæmophilic globulin (as Lister Institute fibrinogen), washed platelets and calcium chloride solution. Each point represents a single reading, and the results are from three separate experiments with the same reagents. Mean saline control clotting time, 23 sec.

The results are shown as ratios of the corresponding saline control clotting times. With each thromboplastic reagent, 0·1 ml. was added to 0·1 ml. of citrated plasma and 0·1 ml. of dextran sulphate solution (prepared from a clinical Injection of dextran sulphate) or saline. Brain and venom were prepared in M/40 calcium chloride solution; in the tests with blood thromboplastin, a further 0·1 ml. of M/40 calcium chloride solution was added with the thromboplastin: the difference in final volumes has been allowed for in plotting the concentrations of dextran sulphate.

iii. *Experiment 7*: *Effect of dextran sulphate on the rate of activation of brain suspension by factors V and VII.* Following Hardisty[9], brain suspension was incubated with diluted serum and a preparation containing factor V, and sub-samples were taken at intervals into mixtures of prothrombin and fibrinogen, and the clotting times recorded. Replicate

runs were made with and without dextran sulphate in the incubated mixture, at $1{\cdot}6 \times 10^{-3}$ g./l. This concentration lay at about the midpoint of the accelerating range in the experiment with purified factors (Experiment 6a, Fig. 5, *Direct* curve), and it was verified that it had a negligible effect on the clotting times of the prothrombin-fibrinogen mixtures, as in Experiment 6b, Figure 5, *Preincubated* curve. The results are shown in Figure 6, where an accelerating effect of the dextran sulphate is clearly seen. There was no effect upon the final activity of the incubated mixtures.

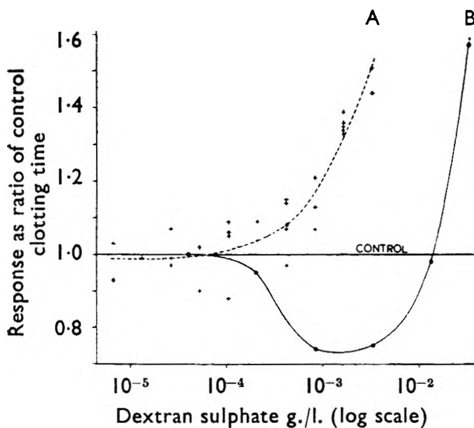

FIG. 5. *Tests with brain suspension and purified clotting factors.* (Experiment 6.) *Direct* curve (B): to 0·1 ml. dextran sulphate solution or saline were added 0·1 ml. quantities of solutions containing factor V, serum, prothrombin and fibrinogen respectively, and finally 0·1 ml. of a brain suspension in M/40 calcium chloride solution. Each point represents the mean of two readings. Mean saline control time, 18·4 sec.

Preincubated curve (A): Brain suspension was activated by incubation for 3 min. with factor V, serum and calcium chloride solutions. The mixture was then transferred to an ice bath and 0·1 ml. aliquots were tested against 0·2 ml. volumes of mixtures of prothrombin and fibrinogen with dextran sulphate solution or saline. Each point represents the mean of 2 readings; the data were derived from 5 batches of activated brain suspension. Mean saline control time, 14·5 sec. (Further points, obtained at lower drug concentrations but omitted from the graph, did not show a significant departure from the control clotting time.)

In each case the results are shown as ratios of the corresponding mean saline control clotting time for each run. Injection of Dextran Sulphate.

These experiments suggest that the prothrombin time is accelerated by a certain range of concentrations of dextran sulphate because under these circumstances the drug hastens the activation of the brain reagent by factors V and VII. Jenkins[16] found that Russell's Viper venom appeared to act as though it possessed the activities both of brain suspension and of factor VII. Figure 4 shows little difference between the acceleration produced by dextran sulphate when brain or venom were used, so that the acceleration would seem not to involve a reaction directly between factor VII and the thromboplastic reagent, but rather the reaction between brain and factor V, postulated by Hardisty[9], of which the rate is governed by factor VII[9].

4. *The role of* N-*sulphate and* O-*sulphate in the Accelerating Effect*

There is evidence that in heparin the sulphate groups are attached both by *N*-[17,18] and by *O*-linkages[19], whereas in dextran sulphate, the attachment is, of course, by *O*-linkages only[20]. It is believed that the *N*-sulphate linkages in heparin may be selectively broken by gentle acid hydrolysis[21],

which yields a derivative of low anticoagulant activity[17] known as ψ-heparin[22].

A series of ψ-derivatives were obtained from heparin after successive periods of acid hydrolysis (1 per cent. w/v solution of heparin in 0·04N HCl at 100° C.). Samples were withdrawn at 8-minute intervals up to

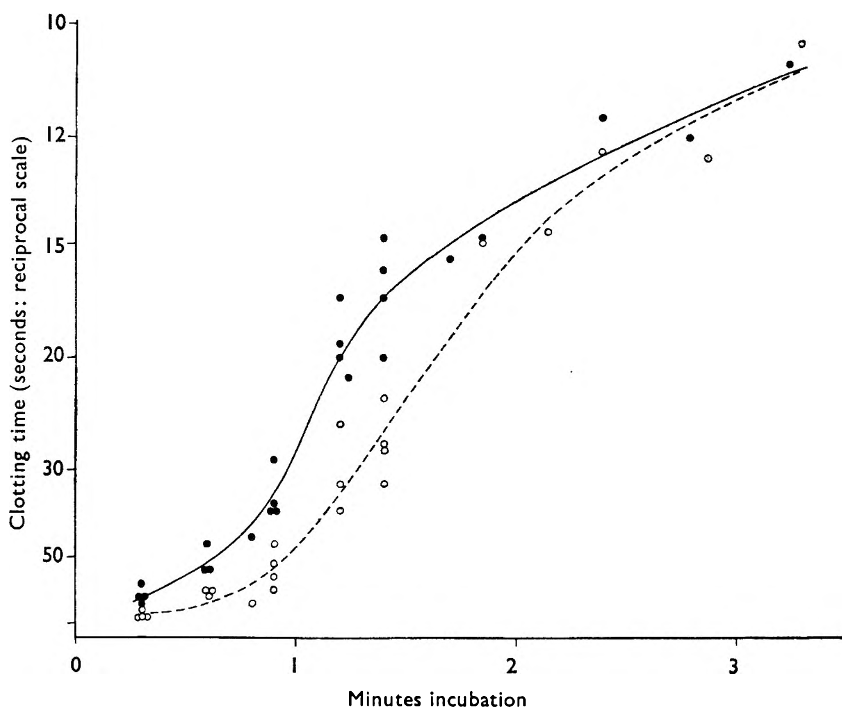

FIG. 6. *The effect of dextran sulphate upon the rate of activation of brain suspension by factors V and VII.* (Experiment 7.) Brain suspension was incubated with serum, a preparation of factor V, calcium chloride solution, and amino-*tris*(hydroxymethyl)-methane buffer, pH 7·3, 0·00625M[15]. The reaction was sampled at intervals into aliquots of a mixture of prothrombin and fibrinogen (following Hardisty[9]), of which the clotting times were recorded, and are shown reciprocally on the ordinate.

Unbroken Line: the incubated reaction contained dextran sulphate (prepared from the Injection), $1·6 \times 10^{-3}$ g./l., added in the buffer.

Broken Line: the incubated reaction contained the buffer only.

The data represent 10 runs, 5 with and 5 without dextran sulphate. The drug concentration tested was chosen to lie at about the mid-point of the acceleration of the *Direct* (B) curve of Figure 5.

48 minutes; the 40-minute sample is not reported because its activity differed little from that of the 48-minute sample. The derivatives were isolated by freeze-drying after neutralisation and dialysis. Half the *N*-sulphate groups had been hydrolysed in *ca.* 20 minutes and all in 90 minutes, so that over the tested range, the derivatives would have retained 40–90 per cent. of the original *N*-sulphate groups (Dr. A. B. Foster, personal communication). Dose-response curves were obtained in the thrombin-plasma clotting time[23] and prothrombin time tests.

Experiment 8: *Dose-response curves in the thrombin-plasma clotting time test.* The 32 and 48 minute samples showed no anticoagulant activity, but dose-response curves obtained in the thrombin-plasma test with the earlier samples and with dextran sulphate are shown in Figure 7, where the ψ-series exhibits a progressive fall in the slope of the curves, passing from the high slope given by the parent heparin towards the low slope characteristic of dextran sulphate[1]. There is also apparent a progressive fall in anticoagulant activity shown by the positions of the curves.

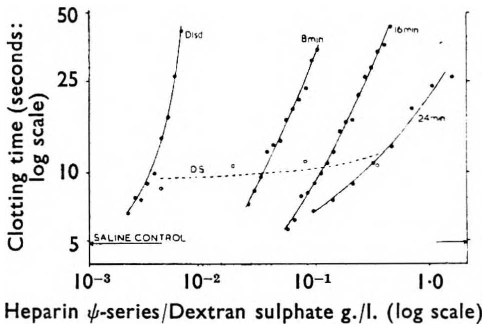

FIG. 7. *Thrombin-plasma clotting times as dose-response curves with dextran sulphate, heparin and hydrolysed heparins (ψ-series).* (Experiment 8.) The concentrations of plasma and thrombin were constant, and gave a saline control clotting time of 4·9 sec.; 0·2 ml. plasma received 0·1 ml. drug solution or saline, followed by 0·1 ml. human thrombin (Lister Institute) dissolved in M/20 calcium chloride solution. Each point represents the mean of 4 replicate readings.

DS: dextran sulphate, British Standard.

Dlsd: the parent heparin, dialysed only.

8 min., 16 min., 24 min.: ψ-samples obtained after these periods of hydrolysis.

Experiment 9: *Dose-response curves in the prothrombin time test.* Similar dose-response curves obtained in the prothrombin time test are shown in Figures 8 and 9. Figure 8 shows the results of a single experiment which determined the positions of the curves given by the various ψ-samples: the findings are similar to those of the thrombin-plasma test. In Figure 9 are shown, separately for each of the last 4 ψ-samples tested, the collected results of a number of experiments in which the accelerating effect was particularly studied. It may be seen that as the anticoagulant potency falls, the acceleration appears and increases progressively along the series, until finally the acceleration alone remains.

As a corollary to these experiments, tests were made with polyvinyl amine sulphate, which is a long-chain molecule carrying *N*-sulphates only; and, for comparison, also with polyvinyl alcohol sulphate, carrying *O*-sulphates only. An acceleration of prothrombin time was not observed with either drug.

DISCUSSION

These experiments have investigated the acceleration of the prothrombin time by a certain range of concentrations of dextran sulphate. The results suggest that this acceleration is effected by hastening the preliminary reaction(s) occurring between the brain reagent and factors V and VII, and that the phenomenon is a function of *O*-sulphate. A comparable phenomenon could not be detected when blood clotted spontaneously, and the acceleration of the prothrombin time test is not therefore thought to be of clinical significance.

Two theoretical possibilities now arise:—

1. The accelerating and the anticoagulant activities of dextran sulphate may depend upon different mechanisms, exerted independently upon the clotting system. Over the anticoagulant range of concentrations both effects might be present together but the acceleration would now be masked by the anticoagulant activity. The observed dose-response curve would thus be the resultant of the two opposing effects, but if the anticoagulant effect could be plotted alone, this curve would be expected to lie further down the drug concentration axis. An anticoagulant curve was in fact observed in this position in the experiments with blood thromboplastin (Experiment 5: Fig. 4) and activated brain suspension (Experiment 6b: Fig. 5, *Pre-incubated* curve) in both of which the reactions involving factors V and VII had occurred before dextran sulphate was added to the system. Similarly, the acceleration plotted alone would be expected to be even greater than that observed in the present experiments, but no method of doing this was devised. This hypothesis is illustrated in the upper part of Figure 10.

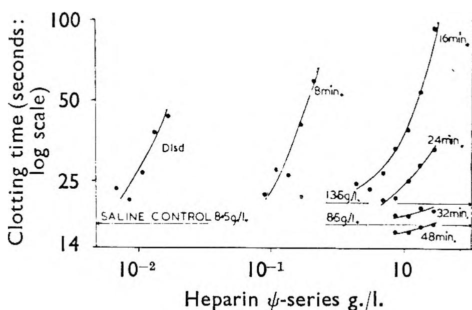

FIG. 8. *One-stage prothrombin times as dose-response curves with heparin and hydrolysed heparins (ψ)-series.* (Experiment 9.) The concentrations of plasma and brain reagent were constant and gave a control clotting time with 0·85 per cent. saline of 17·3 sec.; 0·1 ml. plasma received 0·1 ml. drug solution or saline, followed by 0·1 ml. brain suspension in M/40 calcium chloride solution. Each point represents the mean of 2 replicate readings.

When testing the higher drug concentrations (which were all added to the reaction in saline) it was thought that the total solute concentration might have been high enough to have prolonged the clotting time. A second series of control readings was therefore made with 1·35 per cent. (w/v) sodium chloride solution (to represent 0·85 per cent. (w/v) sodium chloride solution + 0·50 per cent. drug concentration, which was the highest prepared: this yielded the highest concentration on the graph, 0·16 per cent., when diluted with the plasma and brain suspension), and the mean of these readings was 21·1 sec. If this control value is used for comparison with the clotting times obtained with the highest drug concentration plotted, the acceleration produced by the 48 min. sample is still more striking.

Dlsd: the parent heparin, dialysed only.

8 min., etc.: ψ-samples obtained after these periods of hydrolysis.

If this were true, the findings in Experiments 2 and 3 (Figs. 2 and 3) might be explained by supposing that variations in the concentrations of plasma or brain suspension altered the relative position of the accelerating and anticoagulant curves, and so affected the shape of the observed resultant. The data of Experiment 4 (Table I) are clearly also in line with this hypothesis, because they show that the accelerating effect of dextran sulphate can still be observed in the presence of an anticoagulant concentration of heparin.

2. On the assumption that a progressive reduction in *N*-sulphate is

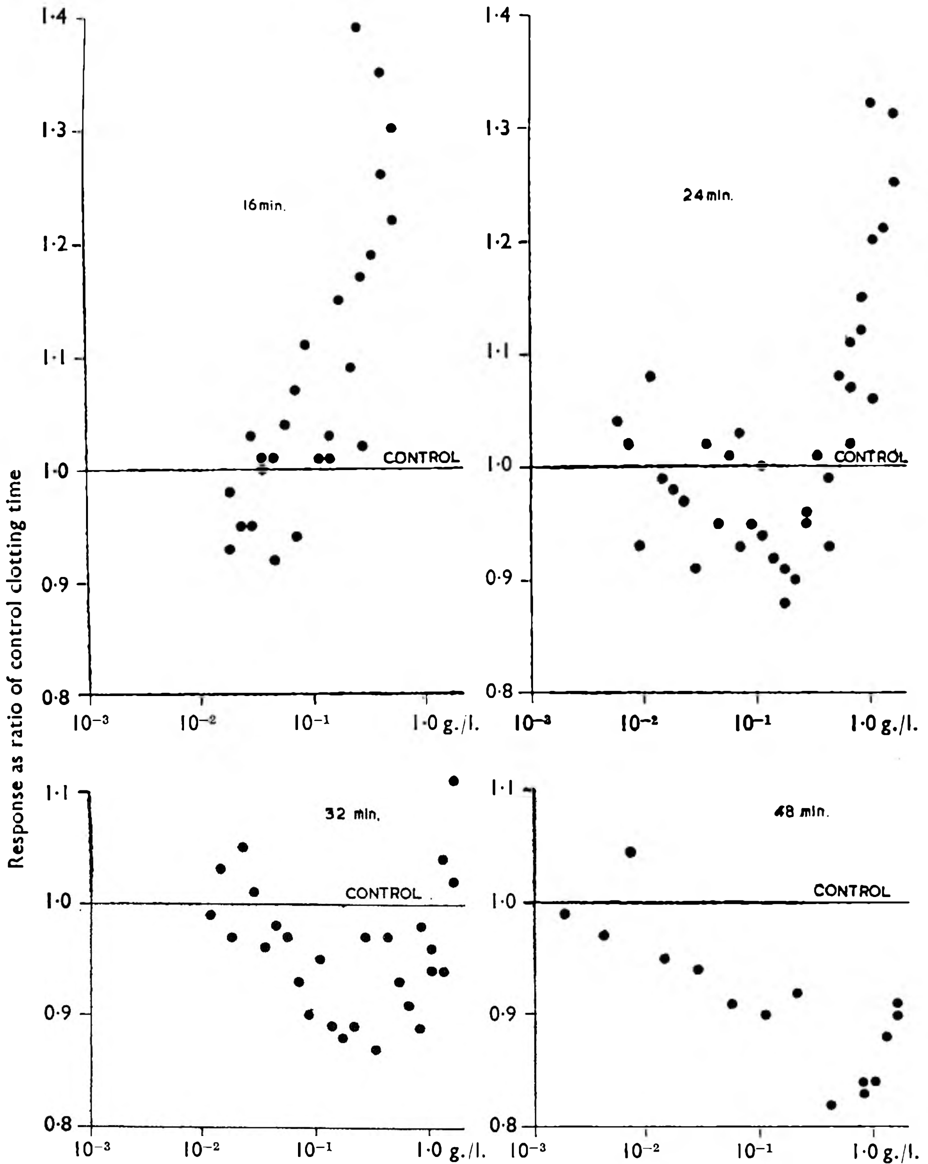

FIG. 9. *The acceleration of the prothrombin time by hydrolysed heparins (the last 4 samples in the ψ-series).* (Experiment 9.) The conditions were as for Figure 8. Each point represents a single reading, and the data are collected from 7 experiments in which the saline control clotting times lay between 18·2 sec. and 21·8 sec. In each case the drugs were added in saline, and the control readings were obtained with a concentration of saline corresponding to the total solute concentration in g./l. 16 min., etc.: ψ-samples obtained after these periods of hydrolysis.

the only change induced in heparin by gentle acid hydrolysis, the experiments with the ψ-derivatives suggest that heparin may possess an homologous biphasic activity, by virtue of its *O*-sulphates. (In fact, some *O*-sulphate linkages may also have been broken (Dr. A. B. Foster, personal communication), which might have the effect of reducing still further the anticoagulant activity of the resulting compound.) Comparison of Figures 1 and 9 shows that the accelerating effect of the ψ-derivatives occurs at a much higher drug concentration (*ca.* 500-fold) than the accelerating effect of dextran sulphate. A comparison between the dose-response curves obtained with untreated heparin (Fig. 1), and with dextran sulphate in the "preincubated" experiments (Figs. 4 and 5), suggests that the purely anticoagulant activities of the two drugs would be of the same order of potency in the prothrombin time test. This hypothesis is illustrated in the lower part of Figure 10, which shows the observed resultant curves for untreated heparin, the dialysed parent sample of the ψ-series and the ψ-derivatives. Clearly, the accelerating effect of untreated heparin would be masked by the anticoagulant effect. It has been suggested that the anticoagulant activity of untreated heparin largely depends upon the *N*-sulphates[20,24]. On this hypothesis, the anticoagulant activity of the *O*-sulphates of heparin might only be observed with higher drug concentrations that have been tested. This would explain why the 48 minutes ψ-sample appears to have no anticoagulant activity.

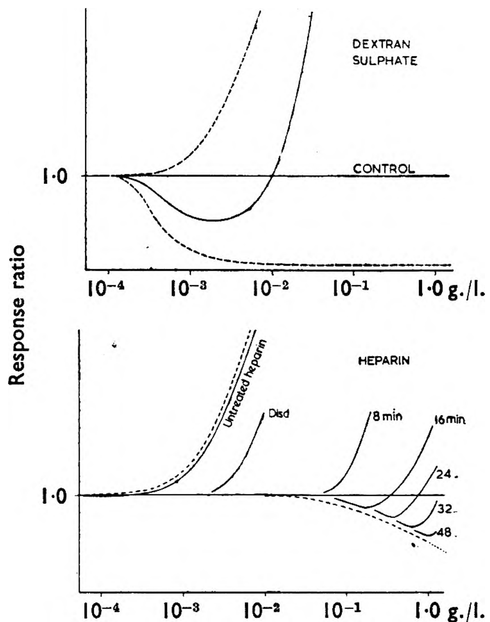

FIG. 10. *Curves illustrating the hypothesis of independent accelerating and anticoagulant activities of dextran sulphate and heparin in the one-stage prothrombin time test.* The full line represent the observed dose-response curves, plotted as ratios of the control clotting times. The broken lines represent the hypothetical anticoagulant and accelerating activities respectively, plotted in the same way. For the dextran sulphate curves, *cf.* Figures 1, 4 and 5. For the heparin curves (which include the observed curves with the ψ-series), *cf.* Figures 1, 8 and 9: data suggesting the dotted portion of the broken curve in the heparin figure were obtained from an independent ψ-sample which accelerated the prothrombin time by about 25 per cent. at the highest tested concentration of 1·6 g./l.

SUMMARY

1. At drug concentrations immediately below the anticoagulant range, the one-stage prothrombin time was accelerated by dextran sulphate to

about 10 to 20 per cent. The magnitude of the effect varied between different batches of reagents, and with variations in the concentrations of plasma and brain suspension. The acceleration could still be detected in the presence of heparin in anticoagulant concentration, and was also seen with Russell's Viper venom.

2. The acceleration was absent, and the anticoagulant potency of dextran sulphate was increased, if the test was made with blood thromboplastin, or with brain suspension activated by preincubation with clotting factors V and VII. Dextran sulphate was found to increase the rate of activation of brain suspension by these factors, and this was thought to explain the acceleration of the ordinary test.

3. The acceleration was not detected in the spontaneous coagulation of blood, and is not therefore thought to be of clinical significance.

4. A similar acceleration was observed with ψ-derivatives of heparin obtained by acid hydrolysis, a process which is believed selectively to break *N*-sulphate linkages but not to greatly affect *O*-sulphate linkages.

5. It is suggested that with both dextran sulphate and heparin there are separate accelerating and anticoagulant effects, and that the observed dose-response curves are the resultants of these opposing activities. On this view the accelerating effect of dextran sulphate operates at slightly lower concentrations than the anticoagulant effect, producing the observed biphasic curve; with heparin, on the other hand, the anticoagulant effect (of *N*-sulphate) is exerted at far lower concentrations than the accelerating effect (of *O*-sulphate), and so with untreated heparin the acceleration is completely masked.

We are indebted to Dr. C. R. Ricketts for his advice on the chemical aspects of this investigation, and for having provided samples of polyvinyl alcohol and amine sulphates; to Prof. M. Stacey and to Dr. A. B. Foster for having provided the series of ψ-heparin samples and the details of their preparation; and to Dr. R. G. Macfarlane for having suggested Experiments 2, 3 and 4.

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THE CHROMATOGRAPHIC PURIFICATION AND ULTRA-VIOLET SPECTROPHOTOMETRIC ESTIMATION OF HYDRASTINE AND BERBERINE IN FLUID EXTRACT OF HYDRASTIS

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Of the three alkaloids known to be present in *Hydrastis canadensis* only hydrastine is used therapeutically, although berberine and canadine have some physiological activity[1]. The B.P.C. 1949 assay procedure for hydrastis has a number of undesirable features. It is a gravimetric procedure and includes small amounts of non-alkaloidal substances not easily removed during purification. The assay is expensive in materials and time. It does not determine hydrastine but the sum of hydrastine and canadine and it does not estimate berberine.

The volumetric methods[2–4] proposed for the assay of berberine in drugs may have certain advantages over the gravimetric procedure but no specific method is available for berberine and hydrastine. Brochmann-Hanssen and Evers[5] described a fluorimetric method for the determination of hydrastine in hydrastis. The fact that berberine occurs in hydrastis in considerable quantities although its pharmacological action is two-fifths that of hydrastine[6] renders necessary a specific method for its estimation. The chemical methods of estimation of berberine are complicated and need the purification of the alkaloid when applied to hydrastis.

This work describes an ultra-violet spectrophotometric method for the identification and estimation of hydrastine and berberine in hydrastis.

Experimental

The ultra-violet absorption spectra of pure samples of hydrastine and berberine hydrochloride have been examined using absolute ethanol as a solvent by the Unicam spectrophotometer. Hydrastine ($C_{21}H_{21}O_6N$, B.P.C. 1949; m.pt. 132° C.) shows a maximum absorption at 297 mμ with E (1 per cent. 1 cm.) 200.

Berberine hydrochloride ($C_{20}H_{18}O_4N,HCl.2H_2O$, B.P.C. 1934) shows two maximas, one at 270 mμ and the other at 350 mμ with E (1 per cent. 1 cm.) 600 [i.e. E (1 per cent. 1 cm.) of pure berberine alkaloid ($C_{20}H_{19}O_5N$) is 694].

The E (1 per cent. 1 cm.) of each alkaloid is plotted against wavelength and the absorption curves are illustrated in Figure 1.

It is clear from Figure 1 that the absorption at 350 mμ is due only to berberine. Therefore in a pure mixture of hydrastine and berberine, only the amount of berberine can be calculated from the extinction measured at 350 mμ. On the other hand, direct spectrophotometric measurement of the extinction due to hydrastine at 297 mμ is difficult

as the absorption due to berberine overlaps that of hydrastine in this region.

For the estimation of hydrastine in hydrastine-berberine mixtures it is necessary therefore to extract hydrastine quantitatively with ether in presence of ammonia. The ether is distilled and the extracted hydrastine is dissolved in absolute ethanol (or 96 per cent.) and adjusted to a known volume. The amount of hydrastine is determined by measuring the extinction at 297 mμ. This spectrophotometric method was compared with the B.P.C. 1949 assay procedure on prepared amounts of hydrastine berberine mixtures. The results are given in Table I.

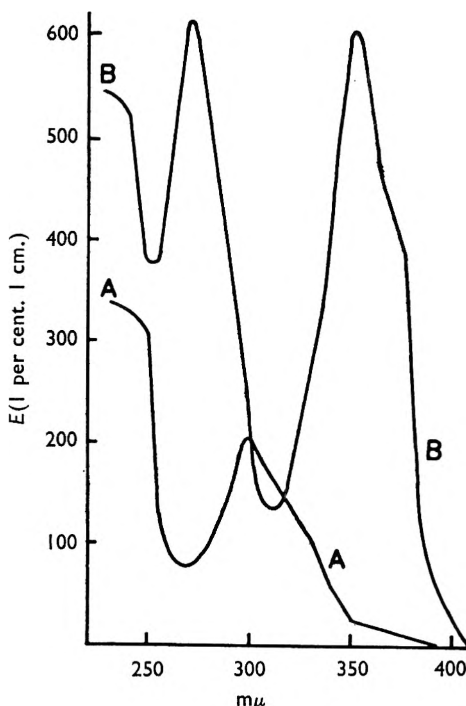

FIG. 1. Showing the absorption of (A) hydrastine and (B) Berberine hydrochloride in absolute ethanol.

Determination of Hydrastine and Berberine in Galenicals

In galenical preparations of hydrastis, berberine always accompanies hydrastine. These alkaloids may be separated by the adsorption chromatographic technique[7,8]. The purified product is then estimated by the spectrophotometric analysis described.

Liquid Extract of Hydrastis. Assay Procedure. Into a glass tube 35 cm. long, 1·5 cm. in diameter with a constricted end, 20 g. of activated alumina was packed dry in portions forming an adsorption column 16 cm. long. The column was connected to a suction apparatus, 1 ml. of the liquid extract added and gentle suction was applied. Before the liquid began to disappear from above the adsorption column, 86 per cent. ethanol was added little by little to wash the alkaloids from the sides of the tube. The adsorption column was then washed with greater amounts of 86 per cent. ethanol until the percolate was alkaloid free. Usually 60 ml. of 86 per cent. ethanol were sufficient for complete washing. The yellow, clear percolate was transferred quantitatively to a 100-ml. flask and adjusted to volume with 86 per cent. ethanol.

For Berberine. An aliquot part of the percolate was diluted (e.g. 1 : 25) with 86 per cent. ethanol and the E 350 mμ value measured. Berberine could be calculated according to the relation $c = E/E$ (1 per cent. 1 cm.).

Where c = concentration in g. per cent.,

E (1 per cent. 1 cm.) = 694 at 350 mμ,

E = measured extinction at 350 mμ.

For Hydrastine. Another aliquot part of the ethanol percolate, usually about 25–50 ml., was distilled on the water bath until a minimum amount of the aqueous liquid remained. About 30 ml. of distilled water was added and the solution transferred quantitatively to a separating funnel.

TABLE I

COMPARISON OF RECOVERY OF BERBERINE AND HYDRASTINE FROM THEIR MIXTURES BY THE SPECTROPHOTOMETRIC AND B.P.C. 1949 METHODS

Spectrophotometric: Prepared amounts of hydrastine-berberine mixture						B.P.C. 1949 gravimetric			
Hydrastine			Berberine			Berberine	Hydrastine		
Used g.	Re-covered g.	Error per cent.	Used g.	Re-covered g.	Error per cent.	Used g.	Used g.	Re-covered g.	Error per cent.
0·0430	0·0420	−2·3	0·0874	0·0899	+2·8	0·478	0·2196	0·2062	−6·1
0·0340	0·0333	−2·0	0·0500	0·0500	0·0	0·402	0·1412	0·1308	−7·3
0·0580	0·0550	−5·1	0·0100	0·0100	0·0	0·685	0·1036	0·0874	−15
0·0133	0·0135	+1·5	0·0680	0·0650	−4·4	0·0995	0·198	0·190	−4·0
0·0400	0·0400	0·0	0·0160	0·0166	+3·7	0·1842	0·1784	0·1677	−5·9
0·0484	0·0465	−3·9	0·02248	0·02166	−3·6	0·1214	0·1198	0·1108	−7·5
0·0250	0·0250	0·0	0·0600	0·0600	0·0	0·1642	0·0988	0·0926	−6·2
0·0524	0·0520	−0·7	0·0174	0·0183	+5·1	0·1425	0·1014	0·0953	−6·0
0·05944	0·0600	+0·9	0·01278	0·01300	+1·7	0·1535	0·1146	0·1069	−6·7
Average error		−1·28			+0·58				−7·1

About 5 ml. of dilute ammonia and 30 ml. of ether were used for washing out the distillation flask. These washings were added to the contents of the separating funnel and hydrastine was extracted by shaking. The ether layer was separated and the aqueous layer was further extracted by shaking with three successive portions, each of 30 ml. of ether. The combined ethereal extract was washed three times each with 30 ml. of distilled water or until alkali free, dried over anhydrous sodium sulphate

TABLE II

COMPARISON OF THE B.P.C. 1949 METHOD FOR THE ESTIMATION OF HYDRASTINE AND THE SPECTROPHOTOMETRIC METHOD FOR THE ASSAY OF HYDRASTINE AND BERBERINE IN LIQUID EXTRACT OF HYDRASTIS

Spectrophotometric								B.P.C. 1949 method			
Hydrastine				Berberine				Hydrastine			
Liq. ext. g. per cent.	Added g.	Re-covered g.	Error per cent.	Liq. ext. g. per cent.	Added g.	Re-covered g.	Error per cent.	Liq. ext. g. per cent.	Added g.	Re-covered g.	Error per cent.
2·15	0·0100	0·0100	0·00	3·06	0·0065	0·0062	−4·6	2·008	0·0676	0·0575	−14·9
2·00	0·00507	0·00500	−1·3	3·25	0·0110	0·0107	−2·7	2·010	0·0670	0·0630	−5·9
2·12	0·00507	0·00520	+2·5	3·07	0·0110	0·01109	+0·81	1·966	0·0338	0·0312	−7·6
2·00	0·00676	0·0070	+3·5	3·25	0·0138	0·0144	+4·3	1·994	0·1064	0·1000	−6·0
2·20	0·00100	0·00100	0·00	3·25	0·0194	0·0198	+2·0	1·984	0·1242	0·1152	−7·2
2·11	0·01352	0·01380	+2·0	3·20	0·0221	0·0220	−0·45	1·994	0·1643	0·1514	−7·8
Average 2·096			+1·1	3·18			−0·10	1·992			−8·2

and distilled. The residue was dissolved in 86 per cent. ethanol and transferred quantitatively to a 25 ml. flask and adjusted to volume with 86 per cent. ethanol. A portion of this ethanolic solution was diluted 1 : 10 with the same solvent, then assayed for its hydrastine content by determining its E 297 mμ value.

The spectrophotometric procedure was carried out on liquid extract of hydrastis as an example of hydrastis galenicals with and without the addition of known volumes of standard solutions of hydrastine and berberine in 86 per cent. ethanol. At the same time the galenical was assayed by the B.P.C. 1949 method with and without the addition of known quantities of hydrastine for comparison. The results are shown in Table II.

Conclusions

The proposed spectrophotometric method appears to be specific for hydrastine and berberine. The accuracy of the method is shown by the high recovery of both alkaloids. The procedure estimates berberine in a galenical preparation of hydrastis with great ease, a fact which makes it a delicate and rapid method for this purpose. Because of the small amount used, the time required for completing the assay is reduced to a minimum without sacrificing accuracy. The method does not necessitate the construction of a standard curve; and complete determination of both hydrastine and berberine in the galenical can be performed within three hours. The proposed assay procedure can measure very minute amounts of each of hydrastine and berberine precisely and satisfactorily.

Summary

1. Spectroscopic data for the ultra-violet absorption of hydrastine and berberine are given.

2. These enable the two alkaloids to be identified and estimated in various drug preparations.

3. The method is rapid and the resulting assay is free from complications.

Our thanks are due to H. Shaker, Ph.D., Chief of Biochemistry Dept. of Research Institute, Ministry of Public Health, for his assistance in reading and checking the absorption curves and data.

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A NOTE ON THE PHARMACOLOGY OF RESERPINE

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The introduction of extracts and concentrates of *Rauwolfia serpentina* Benth. into medicine[1] was followed by the isolation and chemical characterisation of reserpine[2]. A number of careful pharmacological studies on the properties and mode of action of reserpine have now been made[3]. These indicate that the drug produces its characteristic effects primarily by a direct action upon the brain, but that it also influences other tissues and organs, especially those containing contractile elements. In order to throw some light upon these effects and to confirm and extend the observations of others[4–11], we have investigated some of the actions of reserpine upon isolated tissues and organs.

Preparation of Reserpine Solutions

Reserpine was dissolved in an aqueous solution of 10 per cent. ascorbic acid to give a concentration of 2 mg. per ml. The pH of this solution was about 2·5. Immediately before use, the pH was raised to about 4·5 by the addition of small amounts of 5 per cent. sodium bicarbonate solution. The mixture was then diluted with the physiological saline being used to give a final reserpine concentration of 1 mg. per ml. Reserpine precipitated rapidly from the final solution; hence it was necessary to carry out the addition of sodium bicarbonate immediately before use. The control solution was prepared by treating the 10 per cent. ascorbic acid solution in exactly the same fashion. In some experiments, 0·2 per cent. citric acid solution was used as a solvent. These instances are mentioned specifically in the text.

Methods and Results

All drug concentrations, unless otherwise stated, refer to final bath concentrations, expressed as weight of drug per millilitre of bath fluid.

Skeletal Muscle

Frog Rectus Abdominis Muscle. Frogs of either sex were decapitated and pithed. The rectus abdominis muscle was dissected out and set up in an organ bath containing 10 ml. of frog Ringer's fluid (NaCl, 0·65; KCl, 0·014; $CaCl_2$, 0·012; $NaHCO_3$, 0·02; glucose, 0·2 per cent.). The bath was oxygenated and allowed to remain at room temperature. The muscle was stimulated by the addition of submaximal doses of acetylcholine bromide (ACh) or, using potassium-free frog Ringer's solution, potassium chloride solution (KCl) was added to the bath so as to cause a reproducible contractile response. Addition of reserpine solution did not modify the responses to ACh (0·1 μg.) or KCl (2 mg.) in experiments

carried out in August. When similar experiments were carried out in January, different results were obtained. Reserpine (200 μg.) now reduced the responses to 0·1 μg. ACh and itself caused a slow contraction (Fig. 1). The control solution caused a smaller and much slower contraction (Fig. 1). In some experiments, the control solution had no apparent effect, although reserpine still caused a contraction. When reserpine was dissolved in citric acid, it produced a contraction, but it did not influence the response to ACh.

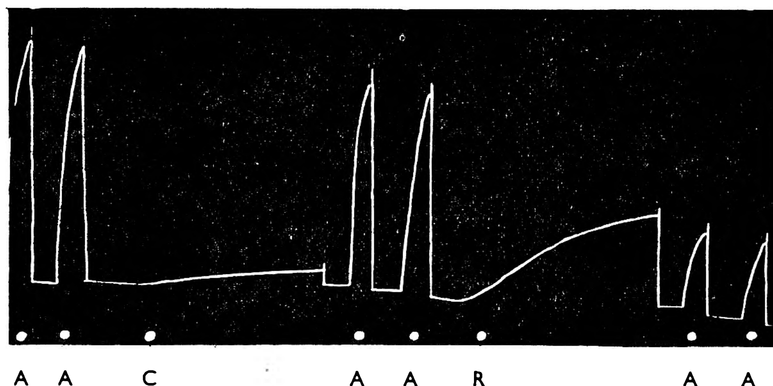

FIG. 1. Direct effect of reserpine on frog rectus abdominis muscle and its influence on ACh induced contractions. At A, 0·1 μg. ACh added. At C, 0·2 ml. ascorbic acid control added. At R, 10 μg. reserpine added.

Cardiac Muscle

Isolated, Perfused Kitten and Rabbit Hearts. The hearts were rapidly dissected out, freed from extraneous tissues and washed in heparinised Locke's solution (NaCl, 0·9; KCl, 0·042; $CaCl_2$, 0·024; glucose, 0·2; $NaHCO_3$, 0·05 per cent.) containing double the usual amount of glucose. They were perfused through the aorta by Langendorff's method[12]. The temperature was maintained at 37° C. The perfusion fluid was well oxygenated. Outflow was measured by a modification of Thorp's impulse counter. Reserpine in a concentration of 0·1 or 1·0 μg. per ml. increased the outflow, in some cases by as much as 100 per cent., but in other experiments, the increase was of the order of 10 per cent. (Fig. 2). There was a slight reduction in heart rate. The amplitude usually underwent a gradual reduction. In most instances, the effects upon amplitude and outflow were partially reversible when reserpine infusion ceased. After perfusion with respine, the heart muscle often showed a loss of tone. The control solution had qualitatively similar, but quantitatively weaker, effects. Reserpine had no significant influence upon the cardio-accelerator action of (—)-adrenaline hydrochloride (5 ng.), (—)-noradrenaline bitartrate* (1 ng.), histamine acid phosphate (Hm) (10 μg.) or 5-hydroxytryptamine creatinine sulphate (5-HT) (10 μg.), but appeared to reduce the duration of their action. Reserpine had no demonstrable effects

* (—)-adrenaline and (—)-noradrenaline were used throughout the work.

upon the characteristic actions of adrenaline, noradrenaline, Hm or 5-HT on cardiac amplitude or outflow. The reduction in outflow after barium chloride and pitressin was antagonised by reserpine. This confirmed the observations of Tripod and Meier[11]. Reserpine did not influence the effect of pitressin and barium chloride upon the heart rate or amplitude.

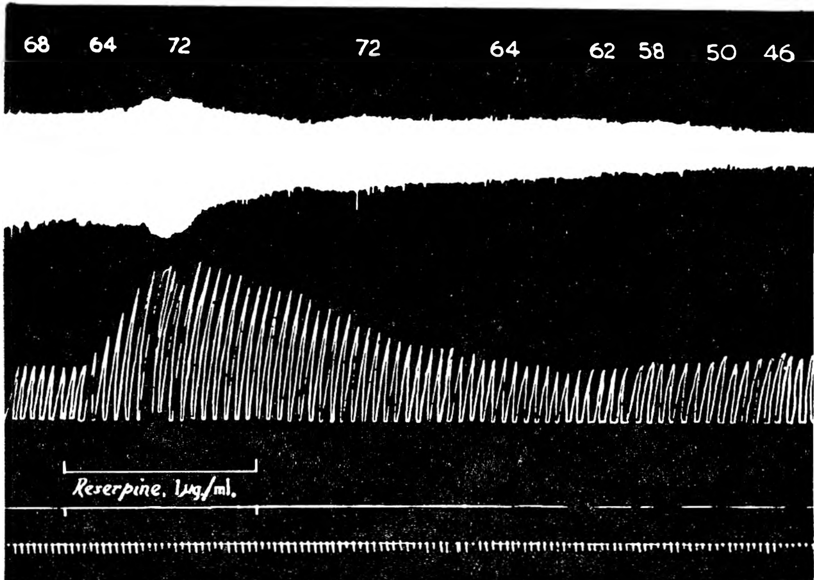

FIG. 2. Influence of 1 μg. per ml. reserpine on amplitude, rate and outflow of perfused kitten heart. Upper record, amplitude (beats per minute); middle record, outflow; lowest record, time = 60 seconds.

The Isolated Guinea-pig and Rabbit Auricles. The auricles were set up in a 25 ml. bath containing well oxygenated Locke's solution at 29° C. Reserpine, 1 μg. or 10 μg., reduced the rate and amplitude of the spontaneous contractions (Fig. 3). The reduction in amplitude was occasionally very marked and occurred soon after addition of the drug, but in general it was gradual and less dramatic. The increases in rate and amplitude produced by Hm (0·5 μg.), adrenaline (0·25 μg.) and noradrenaline bitartrate (0·1 μg.) were reduced, usually reversibly, by 1 and 10 μg. of reserpine added 5 minutes beforehand. The responses to noradrenaline were more actively antagonised than those to adrenaline (Fig. 3). Return to control levels of response was slow (up to 2 hours). A delayed reduction in amplitude was seen after washing out the bath (Fig. 3).

Smooth Muscle

The Isolated Guinea-pig Terminal Ileum. A 3-cm. length of the terminal ileum was removed, washed in Tyrode's solution (NaCl, 0·8; KCl, 0·02; $NaHCO_3$, 0·1; $CaCl_2$, 0·02; $MgCl_2$, 0·001; NaH_2PO_4, 0·005; and glucose, 0·1 per cent.) and set up in a bath containing 2 ml. oxygenated

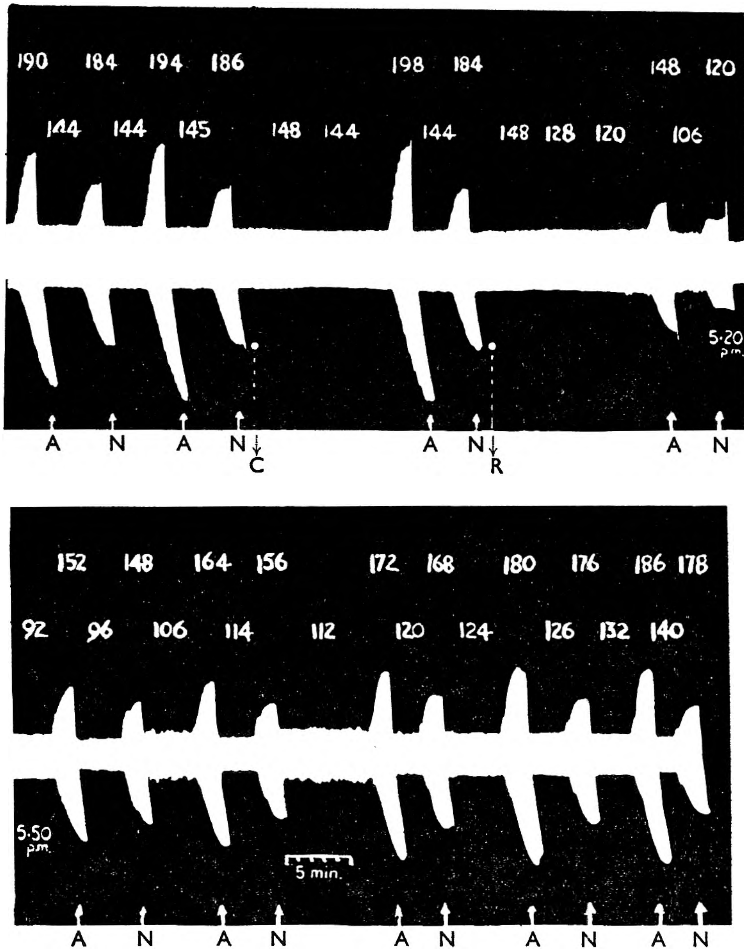

FIG. 3. Influence of reserpine on rate, amplitude and response to adrenaline and noradrenaline of isolated guinea-pig auricles. Figures refer to number of beats per minute; upper row—after drugs; lower row—normal beat (before drugs). At A, 0·25 μg. (−)-adrenaline added. At N, 0·1 μg. (−)-noradrenaline added. At C, 0·1 ml. control solution added. At R, 1 μg. reserpine added. Time = 60 seconds.

Tyrode's solution at 37° C. The responses to submaximal doses of ACh (0·5 μg.), Hm (0·5 μg.), 5-HT (20 ng.) and barium chloride (0·5 mg.) were reduced by doses of from 5 μg. to 30 μg. of reserpine (Fig. 4). The degree of inhibition was related to some extent to the dose of reserpine. Larger doses of reserpine exerted a more prolonged effect than smaller ones, recovery in some cases taking as long as an hour. Maximum inhibition of the response was usually observed after the second or third addition of the spasmogen (Fig. 4). Reserpine itself appeared to have no direct effect upon the ileum. The control solution had no effect. When these experiments were repeated using citric acid solution

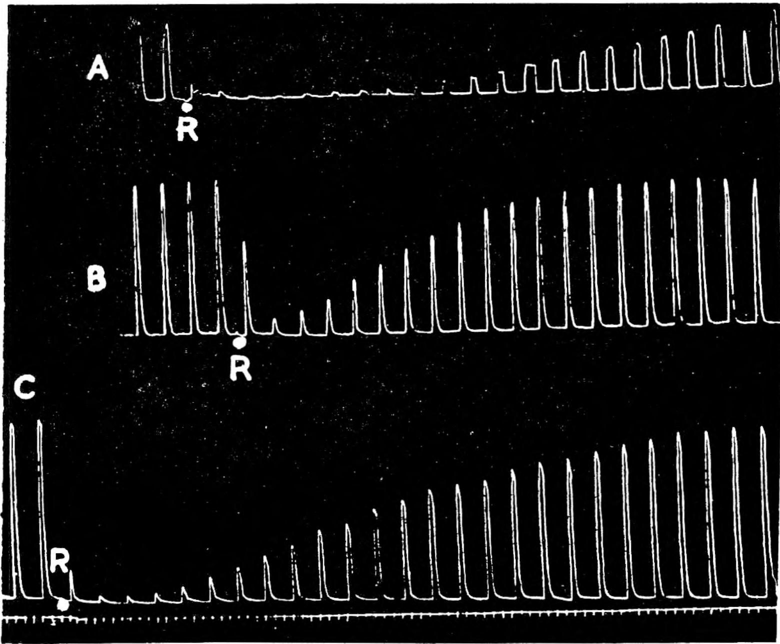

FIG. 4. Influence of reserpine on contractions of guinea-pig ileum induced by Hm, barium chloride and 5-HT. A, All contractions induced by 20 ng. 5-HT. B, All contractions induced by 0·5 μg. Hm. C, All contractions induced by 0·5 mg. barium chloride. At R, 30 μg. reserpine added to bath. Time = 60 seconds.

as solvent, the inhibition of responses of the ileum to ACh and Hm were reduced. Recovery to control levels of response was quicker (Fig. 5). It was decided to see whether citric acid would prevent reserpine from producing its usual inhibitory effects. When citric acid (200 to 800 μg.) was added to the bath with the reserpine, the inhibitory effect of 30 μg. reserpine was markedly reduced (Fig. 6). Doses of citric acid up to

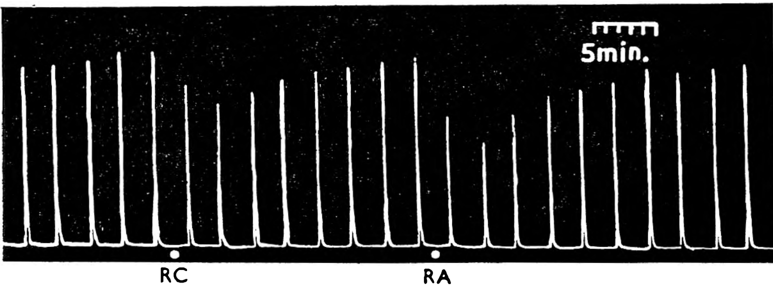

FIG. 5. Influence of reserpine in citric acid solution and ascorbic acid solution on ACh induced contractions of guinea-pig ileum. All contractions induced by 0·5 μg. ACh. At RC, 10 μg. reserpine in citric acid added. At RA, 10 μg. reserpine in ascorbic acid added. Time = 60 seconds.

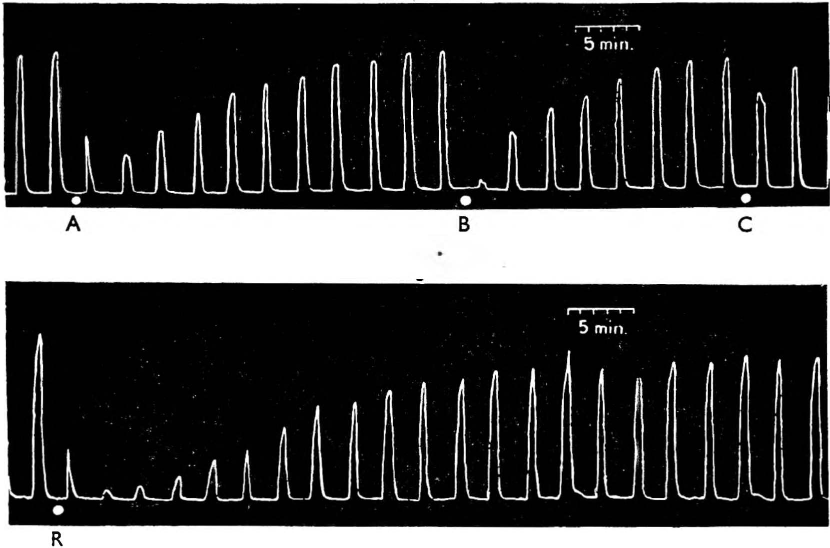

FIG. 6. The influence of added citric acid on reserpine inhibition of ACh induced contractions of the guinea-pig ileum. All contractions induced by 0·5 μg. ACh.
Upper tracing. At A, 400 μg. citric acid with 30 μg. reserpine added. At B, 800 μg. citric acid with 30 μg. reserpine added. At C, 800 μg. citric acid added.
Lower tracing. At R, 30 μg. reserpine added.
Time = 60 seconds.

1·6 mg., however, did not completely prevent the reserpine-induced inhibition. Sodium citrate in doses giving the same bath concentration of citrate ion did not have any inhibitory effect.

The Isolated Rabbit and Kitten Duodenum. About 3 cm. of duodenum were removed, taking a portion which began about 5 cm. distal to the pyloric sphincter. This was set up in a bath containing 5 ml. of oxygenated Locke's solution at 37° C. Reserpine (4 to 30 μg.) inhibited the spontaneous activity of the duodenum (Fig. 7). At the same dose levels,

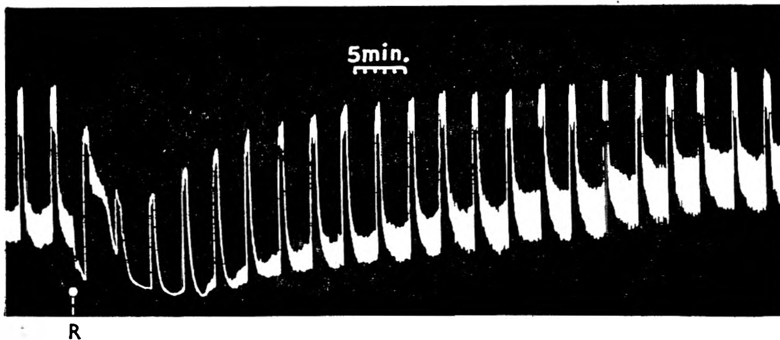

FIG. 7. Influence of reserpine on spontaneous activity and ACh induced contractions of rabbit duodenum. All contractions induced by 0·1 μg. ACh. At R, 30 μg. reserpine added. Time = 60 seconds.

the alkaloid antagonised the spasmogenic action of 0·1 μg. ACh. Reduction in tone caused by 0·5 μg. adrenaline bitartrate was not influenced. The control solution itself showed slight, rather variable, effects upon tone.

Isolated Rat Uterus. Virgin female rats weighing between 120 and 180 g. were brought into œstrus by subcutaneous injections of 0·1 mg. per 100 g. of body weight of stilbœstrol in arachis oil, given 24 hours

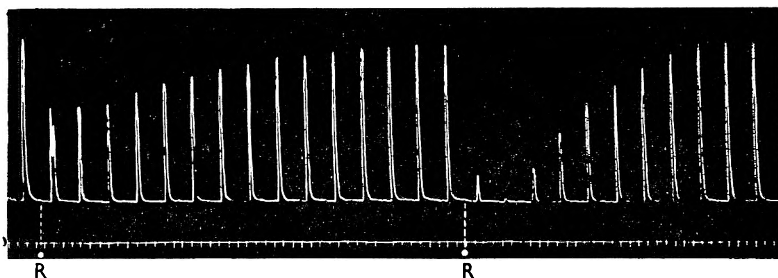

FIG. 8. Influence of reserpine on ACh induced contractions of rat uterus. All contractions induced by 0·25 μg. ACh. At R, 30 μg. reserpine added. Time = 60 seconds.

before use. One horn of the uterus was suspended in a bath containing 2 ml. of oxygenated de Jalon's solution (NaCl, 0·9; KCl, 0·42; $CaCl_2$, 0·006; glucose, 0·05; and $NaHCO_3$, 0·05 per cent.) at 29° C. No direct action was seen at dose levels of from 2 to 100 μg. of reserpine. The initial dose of reserpine reduced (usually reversibly) the responses to KCl (2 mg.), ACh (0·25 μg.) and 5-HT (20 ng.). Similar doses of reserpine, given later, again reduced the amplitude of the response but the effect was now easily reversible (Fig. 8).

The Spinal Cat. Cats of either sex were given atropine (1 mg. per kg.) by intraperitoneal injection 15 minutes before induction of anæsthesia

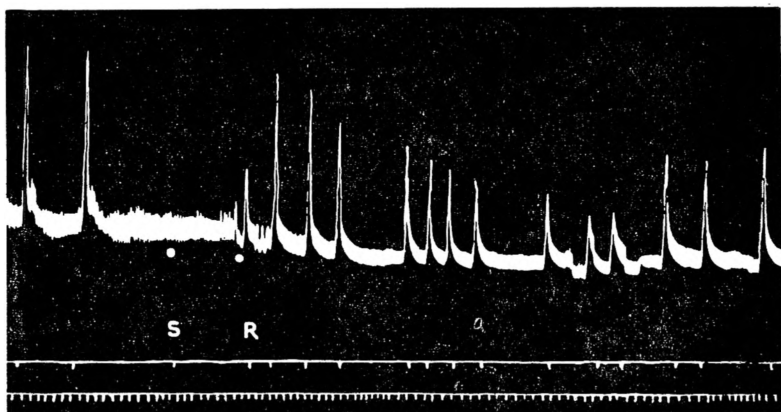

FIG. 9. Influence of reserpine on the pressor response to adrenaline in the spinal cat. All unmarked responses due to 5 μg. adrenaline + 3 ml. saline. At S, 3 ml. saline. At R, reserpine 1 mg. per kg. Time = 60 seconds.

with ether. The carotid arteries were tied and the spinal cord was transected at the level of the second cervical vertebra. The brain was destroyed by a blunt probe. Blood pressure was recorded from the carotid artery and drugs were given by a cannula inserted into the femoral vein. In young cats* a progressive, gradual reduction in blood pressure was noted with 1 mg. per kg. of reserpine. The responses to 5 μg. adrenaline and 1 μg. noradrenaline bitartrate were also progressively reduced (Fig. 9). The effect was not reversible during experiments of 6 to 8 hours. In older animals*, this effect was less marked. Older cats did not show any fall in blood pressure to reserpine. At the point of maximal sustained reserpine-hypotension, Hm (5 to 10 μg.) produced no further fall in blood pressure. Reserpine did not inhibit the pressor response to posterior pituitary extract. In some experiments, however, the latter appeared to reverse the reserpine-induced reduction of the adrenaline response.

Discussion

At the moment, we can offer no explanation for the varying effects of reserpine on the frog rectus abdominis muscle.

The effects of reserpine on the heart—decrease in amplitude with increase in outflow—may indicate a reduction in extravascular support for the coronary bed, that is, a reduced tone of cardiac muscle.

On the smooth muscle of the ileum and uterus, no decrease in tone is evident, but spontaneous activity is inhibited and tone is reduced in rabbit and kitten duodenum. There is a non-specific antagonism shown to the effects of ACh, Hm, HT and barium chloride. This is a prolonged effect and in most instances the maximum effect is delayed in onset.

In the spinal cat, where any vascular tone remained, this was progressively reduced by reserpine to a point where further administration of Hm caused no depressor response. There was antagonism to adrenaline in younger animals but not in older ones. In general, reserpine reduces the tone of cardiac and smooth muscle. Preliminary experiments using the isolated, perfused rabbit ear and rabbit hind quarters indicate that drug-induced vasoconstriction is reduced by low concentrations of reserpine, which themselves have no direct observable effect.

The results obtained indicate that as far as the preparations used are concerned, reserpine does not act upon specific receptors as does, for example, atropine. It may be acting at some point in the metabolic processes underlying contraction—drug-induced or myogenic. This suggestion is supported by the observation that reserpine inhibition is reduced by citric acid. This is now being investigated, as the possibility of an effect upon tissue calcium cannot yet be excluded.

The delay in reaching maximum inhibition may be explained in a number of ways, although experimental proof is lacking. Reserpine may undergo a chemical change to a more active form, or the delay may be related to its low solubility. It is also possible that reserpine is acting as an anti-metabolite.

* Cats of 8 years or over were considered to be "old".

Summary

1. The actions of reserpine have been investigated using a number of tissues containing contractile elements.

2. Both the direct effects of reserpine and its actions upon drug-induced responses have been investigated.

3. Reserpine has been found to antagonise some of the effects of acetylcholine, (−)-adrenaline, (−)-noradrenaline, 5-hydroxytryptamine, pitressin, barium chloride and potassium chloride on such preparations as the frog rectus abdominis muscle, the isolated guinea-pig ileum, and auricles, the isolated perfused heart and the spinal cat.

4. It is suggested that reserpine may affect the metabolic processes which underlie muscular contraction.

We wish to thank Dr. C. Dale Falconer of Ciba Laboratories Ltd., for assisting us in these investigations with gifts of reserpine. We are also grateful to Messrs. Upjohn Ltd., and Messrs. Abbott Laboratories Ltd., for gifts of 5-hydroxytryptamine creatinine sulphate, and we thank Miss I. Topping and Miss J. Hall for technical assistance.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

p-Aminosalicylic Acid, Determination of Small Quantities of. Yu. M. Ostrovskiĭ. (*Aptechnoe Delo*, 1955, **4**, No. 6, 10.) When *p*-aminosalicylic acid (PAS) is oxidised with potassium ferricyanide in alkaline solution, an intense orange-brown colour is produced; the reaction can be used to detect 0·01 mg. of PAS in 1 ml. of solution. For the quantitative determination of PAS, 2 ml. of a 0·005 to 0·05 per cent. solution is treated with 0·6 ml. of a freshly prepared solution of potassium ferricyanide (prepared by mixing 15 ml. of 33 per cent. sodium hydroxide solution with 35 ml. of 2 per cent. potassium ferricyanide solution). After 15 minutes the colour is compared with standards prepared with known amounts of PAS, or the density is measured in an absorptiometer and the PAS content of the sample is calculated with the aid of a calibration curve. The solution is stable for 15 minutes; when ascorbic acid is added the stability is increased but the sensitivity of the reaction is reduced. E. H.

Cycloserine, Colorimetric Determination of. L. R. Jones *Analyt. Chem.*, 1956, **28**, 39.) Cycloserine was found to react with sodium nitritopentacyanoferroate in a slightly acidic medium to give an intense blue complex suitable for quantitative measurement at 625 mμ. The colour developed rapidly and was stable for several hours, deviating slightly from Beer's law but being reproducible in the range of 5 to 200 μg. of cycloserine. Several variables were investigated for their influence on the colour formation including the absorption curve, stability and intensity of colour, time and temperature of reaction, stability and concentration of sodium nitritopentacyanoferroate, acid-base concentration, and interference from other compounds. The reagent must be prepared freshly for each set of determinations, two stock solutions of 4·0N sodium hydroxide and 4 per cent. aqueous sodium nitroprusside being prepared, mixed, and used within 15 minutes for each analysis of cycloserine. The blue complex showed a maximum absorption at 625 mμ, the highest precision and reproducibility being obtained at a temperature of 25° ± 1° C. Cooling samples below 15° C. resulted in slow colour formation; heating samples above 50° C., completely destroyed the colour. Interference was found only with derivatives of cycloserine which still retain the basic ring structure, although low recoveries were obtained on urines containing added cycloserine. R. E. S.

Digitalis Glycosides in Chemical Assay. F. H. L. van Os and D. H. E. Tattje. (*Pharm. Weekbl.*, 1955, **90**, 901.) Among recently discovered glycosides of digitalis, strospeside (gitoxigenin + digitalose) is important since it is stated to correspond to about one half of the digitoxin content, or one tenth of the total glycosides. Chemicai assay of digitalis by the Baljet reaction (sodium picrate) determines this glycoside, but not the inactive digipurpurin. On the other hand methods of assay based on digitoxose will determine digipurpurin, and therefore give misleading results. The compounds which react to the Baljet reaction are digitoxin, gitoxin, gitaloxin, strospeside, gitorin, with the minor constituents odoroside H, diginin and digifolein. Thus by this method there is some promise of an ultimate agreement between chemical and biological assays. G. M.

Vitamin B_{12} in Liver Injection, A Chemical Method for. P. J. VanMelle. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 26.) Samples of injectable liver extracts containing 120–250 μg. of vitamin B_{12} were treated with potassium cyanide at pH 7·5 to convert all cobalamins to cyanocobalamin. The solutions were mixed with citrate buffer, pH 4 and passed through columns of Amberlite XE97 (carboxylic cation-exchanger) in hydrogen form and of suitable particle size to maintain a sufficient rate of flow. Impurities were washed from the column with 0·1 N hydrochloric acid, acetone (85 per cent.) and again with 0·1 N hydrochloric acid. Cyanocobalamin was eluted from the column with dioxan (60 per cent. in water) containing sufficient hydrochloric acid to make it 0·1 N. Two equal 4-ml. quantities of the eluate of each column were taken, one being treated with potassium cyanide (10 per cent.) and the other with alkaline buffer to obtain a similar pH. The solutions were allowed to stand for 20–30 minutes and the difference in absorbancy measured at 578 mμ. The quantity of cyanocobalamin in each sample was calculated from the datum *E* (1 per cent. 1 cm.) difference = 60 for cyanocobalamin. Results obtained were in agreement with those of the microbiological assay using *Lactobacillus leichmannii*, but the chemical method was more precise. Preparations containing more than 100 mg. of solids per μg. of vitamin B_{12} required preliminary purification before the method could be applied. G. B.

GLYCOSIDES, FERMENTS AND CARBOHYDRATES

Diginatin, a New Cardioactive Glycoside from *Digitalis lanata*. J. E. Murphy. (*J. Amer. pharm. Ass., Sci. Ed.*, 1955, **44**, 719.) Diginatin was separated from other glycosides of *Digitalis lanata* by partition chromatography on a Celite column. A mixture of 2 parts of water and 1 part of formamide was used as the stationary phase, and chloroform saturated with the stationary phase was employed as the mobile solvent. Digitoxin, gitoxin and digoxin were obtained in the early fractions and diginatin in the later fractions. The glycoside was further purified by chromatography and crystallised from ethanol. It was soluble in ethanol and in dioxan, and slightly soluble in acetone. Solubility in water was about 1 in 1000 and in chloroform about 1 in 2000. On hydrolysis an aglucone ($C_{23}H_{34}O_6$) was obtained together with 3 molecules of digitoxose. The aglucone appeared to be isomeric with digoxigenin and gitoxigenin, except that it had one extra hydroxyl group. G. B.

Lanatoside ABC, Separation and Determination of Individual Lanatosides and Desacetyllanatosides in. O. Hrdý, Z. Jung and A. Šlouf (*Českoslov. Farm.*, 1955, **4**, 395.) The lanatosides and desacetyllanatosides in lanatoside ABC and commercial lanatoside C are determined by descending paper chromatography. For the separation of lanatoside A, Whatman No. 1 paper is used. The paper is impregnated with formamide and the chromatogram is developed with formamide-saturated chloroform as the mobile phase. Lanatoside B, desacetyllanatoside A, lanatoside C and the desacetyllanatosides B and C are separated on Whatman No. 4 paper, similarly impregnated, a mixture (8:2) of chloroform and ethyl acetate saturated with formamide being used as mobile phase. For evaluation, the spots are cut out and treated with xanthhydrol reagent (0·01 per cent. of xanthhydrol and 1 per cent. of concentrated hydrochloric acid in glacial acetic acid); the extinction of the solution obtained is measured in a Pulfrich photometer with a S 53 filter. A determination requires 100 μg. of sample and the precision is $\pm$ 5 per cent. Results obtained on various commercial preparations are given. E. H.

ORGANIC CHEMISTRY

Hypertension Peptide, Composition of. W. S. Peart. (*Nature, Lond.*, 1956, **177**, 132.) Quantitative study on the composition of the homogeneous pressor peptide (hypertensin or angiotonin) made by the action of rabbit renin on ox serum has shown the empirical structure to be: one residue—leucine, phenyl alanine, tyrosine, proline, aspartic acid and arginine: two residues—valine and histidine. This gives a minimum molecular weight of 1445. A. H. B.

TOXICOLOGY

Barbiturate Poisoning, Acute. P. M. G. Broughton, G. Higgins and J. R. P. O'Brien. (*Lancet*, 1956, **270**, 180.) Blood barbiturate levels have been determined, by a spectrophotometric method, in thirty-six instances of acute barbiturate poisoning. For each barbiturate the blood-level appeared to be related to the degree of consciousness. Blood-levels of the long-acting drugs were higher, and the rates of fall slower, than those of the short-acting ones. Patients who had taken short-acting barbiturates usually recovered more rapidly. Picrotoxin did not influence the rate of fall of the blood-barbiturate levels, but there was a return to consciousness at slightly higher blood-levels. Analysis of gastric contents and washings in six cases showed less than 4 per cent. of the dose to be left in the stomach. Urine analysis showed no correlation between urine levels and the degree of unconsciousness. It is concluded that blood-levels of 9 mg. per 100 ml. of long-acting, 6 mg. of intermediate and 4 mg. of short-acting barbiturates indicate severe poisoning. G. F. S.

Barbiturates, Toxicological Detection of, by Paper Chromatography. M. Ledvina, B. Chundela, B. Večerek and K. Kácl. (*Českoslov. Farm.*, 1955, **4**, 386.) For the detection of barbiturates in urine, a 2 to 10-ml. sample is acidified and extracted with ether; the ether extract is evaporated to dryness and a solution of the residue in ethanol is deposited on Whatman No. 1 paper. The chromatogram is developed with a mixture (2:1) of *n*-pentanol and 20 per cent. ammonia solution. With this solvent extraneous materials present in the ether extract of the sample do not interfere. The descending technique is mainly used. The spots are detected by spraying the paper with 0·5N sodium hydroxide solution and photographing the chromatogram in ultra-violet light at 254 mμ; the light source is a mercury lamp screened with a Jena UG5 and a chlorine filter. The sensitivity of the method is 10 μg. for barbitone, phenobarbitone and phenylmethylbarbituric acid, and 20 μg. for cyclobarbitone, allobarbitone, amylobarbitone, hexobarbitone, 5-allyl-5′-*iso*propyl- and 1-methyl-5-*iso*propyl-5′-bromallylbarbituric acid. E. H.

PLANT ANALYSIS

η-Tocopherol (7-Methyltocol): a New Tocopherol in Rice. J. Green and S. Marcinkiewicz. (*Nature. Lond.*, 1956, **177**, 86.) The lipid fraction was extracted from ground whole Japanese rice with ether. After the usual purification by alkaline saponification and treatment with floridin earth, the vitamin E complex was analysed by two-dimensional chromatography. The papers, after spraying with ferric chloride-dipyridyl reagent in ethanol, showed three well-defined spots in the α, ϵ and ζ-positions; γ and δ-tocopherols were absent. The spot on the ϵ-position, on spraying with diazotised *o*-dianisidine in alkaline solution, showed a strong positive reaction, given only with tocopherols

having a free non-methylated 5-position (the known ε-tocopherol does not react with this reagent). The new spot was shown to be produced by 7-methyltocol (named η-tocopherol) because 1 mg. of the substance was isolated and methylated with formaldehyde, hydrochloric acid and stannous chloride to yield a product which upon examination by two-dimensional paper chromatography gave two new spots identified by R and R_F values and chemical tests as α and ζ-tocopherols. η-Tocopherol has an absorption maximum at 293 mμ [E (1 per cent., 1 cm.), 78], gives a yellow nitroso derivative with nitrous acid, and upon oxidation with nitric acid gives a red *o*-quinone with absorption maximum at 477 mμ [E (1 per cent., 1 cm.), 56]. Rice contains α- ζ- and η-tocopherols in the proportion 47:26:27. A. H. B.

***Artemisia absinthium*, Bitter Principles of.** G. Schenck and N. E. Schuster. (*Arch. Pharm. Berl.*, 1956, **289**, 1.) The substance known as absinthin is actually a mixture of four compounds, which may be separated by chromatography on alumina. These show the following characters:

	Artamarin	Artamarinin	Artamaridin	Artamaridinin
M.pt. °C.	95–96	72	Oil	82
Ultra-violet maximum—mμ	275–280	268–273	275–280	no maximum
Maximum/minimum.. ..	1·27	1·02	2·12	—
Bitter value	850	4450	6800	125000
Ultra-violet fluorescence ..	strong blue	bright yellow	yellow	pale blue

The Table below gives the amount of the different compounds extracted from the drug

Material	Solvent	Bitter substance	Per cent. of dry material
Commercial tincture ..		Artamaridin Artamarinin	0·15 0·02
Sun-dried herb	Ethanol (60 per cent.)	Artamaridin Artamarinin	0·31 0·025
Commercial drug ..	Ethanol (60 per cent.)	Artamaridin Artamarinin	0·15 0·018
Fresh herb	Ethanol (60 per cent.)	Artamaridin Artamarinin	0·15 0·01
Commercial herb ..	Water	Artamarin Artamaridin Artamaridinin Artamarinin	0·025 0·014 0·025 0·08
Fresh herb	Water	Artamarin Artamaridin Artamaridinin Artamarinin	0·023 0·007 0·018 0·06

Other results show that on drying the amount of bitter substances increases. The bitter value of the drug runs parallel with the artamarin content. G. M.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Azovan Blue, Determination of, in Blood and Tissues. D. F. Clausen and N. Lifson. (*Proc. Soc. exp. Biol. N.Y.*, 1956, **91**, 11.) A 5 g. sample of the dye-containing tissue is homogenised in 100 ml. of a concentrated solution of urea (equal parts of urea and water). The homogenate is poured into a flask

and cooled in a cold water bath for five minutes. Two hundred ml. of acetone AR is added and the mixture shaken. Ten ml. of Somogyi reagent I (10 per cent. zinc sulphate 7 H_2O) is added, shaken and followed by 10 ml. of Somogyi reagent II (0·5 N sodium hydroxide). The mixture is shaken for ten minutes and then filtered through a Whatman No. 42 filter paper. The optical density of the filtrate is measured in a colorimeter at 620 mμ. Comparisons are made between samples of tissue plus unknown dye, tissue plus known dye and tissue without dye. For blood use 0·5 to 2 ml. of whole blood, 2 ml. of urea solution, 8 ml. of acetone and 0·5 ml. of each of the Somogyi reagents. The supernatant solution after centrifuging is taken for colorimetry. The method has been used to study the fate of the dye after intravenous injection into rats.

G. F. S.

Plasma and Red Blood Cell Cholinesterase Activity, Determination of. K. B. Augustinsson. (*Scand. J. clin. lab. Invest.*, 1955, **7**, 284.) A method for the routine analysis of whole blood cholinesterases, using thiocholine esters, is described. 0·05 ml. of whole blood is dried on a filter paper at room temperature. The blood spot is cut from the paper and placed in small pieces in a 10 ml. Erlenmeyer flask. For the plasma butyrylcholinesterase assay 1·5 ml. of barbital buffer (sodium barbitone 4·1236 g., potassium acid phosphate 0·5446 g., potassium chloride 44·73 g. and magnesium chloride 0·2665 g. in 1 litre of water) is added; and for the erythrocyte acetylcholinesterase assay 1·4 ml. of barbital buffer and 0·1 ml. of a solution of 10-(α-diethylamino-propionyl)-phenothiazine ($3{\cdot}3 \times 10^{-3}$ M). The mixture is shaken for 15 minutes at 25° C. in a water thermostat and then 0·5 ml. of the substrate solution is added (butyrylthiocholine iodide or acetylthiocholine iodide 0·1 M solutions diluted with 4 parts of barbital buffer before use). The reaction mixtures are shaken at 25° C. for 30 minutes when the reaction is stopped by the addition of 4 ml. of 10 per cent. trichloroacetic acid. The mixtures are filtered and the precipitate washed with 4 ml. of 2 per cent. trichloroacetic acid To the filtrates add 1·0 ml. of 0·01 N potassium iodate, 2 drops of 5 per cent. potassium iodide and 3 drops of 0·5 per cent. starch solution and titrate with 0·005 N sodium thiosulphate. If A = ml. of thiosulphate required to reduce excess iodine then μmoles of substrate hydrolysed = (m.equiv. KIO_3 −m.equiv. $Na_2S_2O_3$) 1000 = ($1{\cdot}00 \times 0{\cdot}01 - A \times 0{\cdot}005$) 1000. The results agree with standard manometric procedures and show an accuracy of ± 3 to 5 per cent.

G. F. S.

PHARMACY

NOTES AND FORMULÆ

Carbopol 934, Pharmaceutical Uses of. B. Misek, J. Powers, J. Ruggiero and D. Skauen. (*J. Amer. pharm. Ass., Sci. Ed.*, 1956, **45**, 56.) Carbopol 934 is a high polymer containing a large proportion of carboxyl groups. A 0·5 per cent. solution, neutralised to about pH 7 by the addition of sodium hydroxide or carbonate is a satisfactory suspending agent for pine oil, coal tar solution, ichthammol and precipitated sulphur. Coarse emulsions may be prepared by using the 0·5 per cent. neutralised solution, or by mixing the oil with the powder and triturating with water, added gradually. They are improved by passing through a homogeniser. Liquid paraffin emulsions are improved by the inclusion of 0·5 per cent. of Tween 40. Carbopol 934 is not a satisfactory suspending agent for benzoic, salicylic and acetylsalicylic acids, zinc oxide, bismuth carbonate, calamine and substances which yield polyvalent metallic ions.

G. B.

Poliomyelitis Vaccine, Preservation of, with Stabilised Thiomersal. E. O. Davisson, H. M. Powell, J. O. MacFarlane, R. Hodgson, R. L. Stone and C. G. Culbertson. (*J. Lab. clin. Med.*, 1956, **47**, 8.) The presence of thiomersal as a preservative in some poliomyelitis virus vaccines has caused a deterioration in antigenicity, particularly when subjected to increased temperatures during storage. This has now been found to be due to an instability of thiomersal in the presence of traces of cupric ions, the degradation products destroying the antigenicity. The addition of the trisodium salt of ethylenediaminetetra-acetic acid (EDTA), which chelates the cupric ions, stabilises the thiomersal, while the antibacterial and antifungal activities of the thiomersal are not diminished. Trivalent poliomyelitis virus vaccine produced in monkey kidney tissue culture may be satisfactorily preserved with thiomersal in the presence of EDTA. EDTA does not prevent the loss of potency of a vaccine which has excess formaldehyde and thiomersal presumably due to a sensitisation of the virus particle to formaldehyde by the very low concentrations of ethyl mercuric ions formed in the dissociation of the mercurial. It is necessary therefore to neutralise excess formaldehyde in the poliomyelitis vaccine with sodium bisulphite. G. F. S.

Pyrogen Tests, Effects of Drugs on. H. H. Frey, G. Holtz and K. Soehring. (*Arch. pharm. Berl.*, 1956, **289**, 29.) The ordinary pyrogen test cannot be used indiscriminately for testing all injection solutions, since certain drugs are able to suppress the pyrogen reaction. To assume that this will also occur with the patient is hardly justified, in the absence of further information. When testing such solutions, e.g., rutin, the effect on the pyrogen sensitivity should be checked. A possible alternative is to use the leucocyte reaction of Todd and others for determining freedom from pyrogens. In testing drugs for their effect on the pyrogenic reaction, Pyrifer may be used with advantage, as its administration at three day intervals does not give rise to any tolerance. G. M.

PHARMACOLOGY AND THERAPEUTICS

Cardiac Glycosides, Determination of the Duration of Action of. M. Rand and A. Stafford. (*Nature, Lond.*, 1956, **177**, 278.) Cardiac glycosides markedly potentiate the action of injected adenosine, in causing a transient period of partial heart block in the guinea-pig (Rand, and others, *J. Pharmacol.*, 1955, **114**, 119). A method for determining the duration of action of cardiac glycosides is now described. Injections of adenosine are made at regular intervals, and then a dose of the cardiac glycoside is given. With a single injection of digoxin the effect persists for longer than 3 hours, but with digoxigenin the effect is only transient. The rate of elimination of digoxigenin is approximately 1 to 1·5 mg./kg./hr. The rate of elimination of other cardiac glycosides is being studied. G. F. S.

Chloroethylamine in the Treatment of Hodgkin's Disease. L. F. Larionov. (*Brit. med. J.*, 1956, **1**, 252.) A new chloroethylamine, namely, 2-chloropropyl-i-(2-chloroethyl) amine hydrochloride (Novoembichin) has been widely used since 1952 in the U.S.S.R. and has now replaced mustine. It has a stronger effect than mustine on hæmopoiesis and a milder effect on the bone marrow. It also has a milder side-effect on the gastro-intestinal tract. It is given by intravenous injection three times a week, from 8 to 16 injections being necessary.

For the treatment of lymphoid leukæmia a dose of 8 mg. is used and for myelogenous leukæmia 10 mg. Injections are continued until the leucocyte count falls to 2500–3000/c.mm. If this fails to produce complete regression of lymph nodes an additional course of treatment is given 6 weeks later. About 300 patients have been treated with the drug over the last 4 years. Immediate positive results were obtained in nearly all cases. These included a decrease in size of the affected nodes or their complete regression, disappearance or amelioration of general symptoms, such as fever and pruritus, and partial or complete recovery of working capacity. The worst results were obtained in patients in the later stages of the disease, particularly where there was involvement of the retroperitoneal nodes. In spite of good immediate results, relapses occurred in many cases. It is important to repeat the treatment at the onset of relapse when symptoms first appear; if treatment is delayed the next stage of the illness sets in. Provided that the treatment is given in the early stages, positive remote results, namely, preservation of life and working capacity for more than 5 years from the beginning of treatment, may be obtained in 50 per cent. of cases. The immediate and late results of the treatment of early cases are at least as good as those of X-ray therapy. Useful results may be obtained, however, by combining the two treatments as follows. (1) an initial course of chemotherapy is given, followed after an interval of 6 to 8 weeks by X-irradiation of nodes which have not completely regressed; or (2) the two methods of treatment are applied alternately in subsequent relapses. More recently, a new drug which is effective orally has been developed; this is 2:6-dioxy-4-methyl-5-(2-chloroethyl)aminopyrimidine (Dopan). This is given in a dose of 8 to 10 mg. twice weekly, and clinical trials indicate that the immediate results are as good as those with the intravenous drug. S. L. W.

Levallorphan and Alphaprodine in Anæsthesia. F. F. Foldes, E. Lipschitz, G. M. Weber and M. Swerdlow. (*J. Amer. med. Ass.*, 1956, **160**, 168.) In a new technique the short-acting analgesic, alphaprodine, was used in combination with the narcotic antagonist, levallorphan, for the supplementation of nitrous oxide-oxygen-thiopentone sodium anæsthesia. The results in 852 patients controlled with the new technique were compared with those in 756 patients in whom nitrous oxide-oxygen-thiopentone sodium was supplemented by alphaprodine alone. The dose of levallorphan was 0·02 mg./kg. of body weight and that of alphaprodine 1 mg./kg. except in older, debilitated patients to whom one-half or two-thirds of these doses were given. The drugs were injected individually before administration of the anæsthetic, the injection of levallorphan preceding that of alphaprodine by 3 to 6 minutes. If the depth of anæsthesia was inadequate at the start of surgery, additional 5 to 20 mg. doses of alphaprodine were given, 2 to 3 minutes apart, until the desired level of anæsthesia was obtained. When this could not be accomplished without depression of the respiratory rate below 12 respirations per minute additional doses of 25 to 50 mg. of thiopentone were given 2 or 3 minutes apart. When the duration of surgery exceeded 2 to 3 hours, or when the patient's spontaneous respiratory activity was inadequate at termination of anæsthesia, one or more additional 0·4 to 0·6 mg. doses of levallorphan were administered. The administration of additional 5 to 20 mg. doses of alphaprodine was governed by the signs of lightening of anæsthesia, the average interval between supplementary doses being from 8 to 20 minutes. The mg./minute requirements of thiopentone were significantly decreased and the mg./minute requirements of alphaprodine significantly increased in the series of patients who received alphaprodine and levallorphan compared to the patients in the control

series. In 92 (27 per cent.) of 344 patients on whom extraperitoneal surgery was performed satisfactory operating conditions could be obtained by alphaprodine and levallorphan without the use of thiopentone. In both series the mg./minute thiopentone and alphaprodine requirements were inversely proportional to the duration of anæsthesia. Levallorphan antagonised the respiratory depressant effects of alphaprodine to a considerable degree without causing any apparent diminution of analgesic activity. No major post-anæsthetic complications were encountered. The incidence of minor post-operative complications was not markedly different in the two series. (The original paper should be consulted for full details of the technique). S. L. W.

2-Methyl Hydrocortisones, A New Series of Steroids. G. W. Liddle and J. E. Richard. (*Science*, 1956, **123**, 324.) An initial study is reported of 2-methylhydrocortisone and 2-methyl-9-α-fluorohydrocortisone in comparison with hydrocortisone and 9-α-fluorohydrocortisone in normal human subjects, in patients with Addison's disease and in adrenalectomised dogs. In humans, oral administration of single doses of methyl-9-α-fluorohydrocortisone (0·025 to 1·0 mg.); 9-α-fluorohydrocortisone (0·2 to 1·0 mg.); methyl hydrocortisone (10 to 400 mg.) and hydrocortisone (100 mg.) caused sodium retention and potassium loss. Assays in dogs showed the 2-methyl compounds to be many times more potent than their non methylated analogues and methyl fluorohydrocortisone to be more potent that aldosterone in sodium retention. The steroids increased absorption of sodium by the renal tubules. The decrease in circulating eosinophils as an index of "glucocorticoid" activity in both man and dog, showed the methylated steroids to be only slightly more potent than the non methylated compounds during the first four hours of treatment, but to have much longer durations of action. It is suggested that the 2-methyl group alters the susceptibility of the steroids to enzymatic attack. After administration of the methyl fluorohydrocortisone, only 5 per cent. of the dose could be accounted for as 17:21-dihydroxy-20-ketosteroids in the urine compared with 30 per cent. for the unmethylated compound. G. F. S.

Pethidine; Controlled Analgesia with Continuous Drip. H. M. Ausherman, W. K. Nowill and C. R. Stephen. (*J. Amer. med. Ass.*, 1956, **160**, 175.) This is a report on 1000 anæsthetic administrations. In 800 the anæsthetic was thiopentone sodium, nitrous oxide-oxygen and pethidine drip, and in 200, serving as controls, thiopentone sodium and nitrous oxide-oxygen only. In all patients thiopentone sodium in a 2 or 2·5 per cent. solution was used for induction and employed intermittently during maintenance. Nitrous oxide-oxygen in a 75:25 ratio was given by semi-closed circle absorption or non-rebreathing techniques, and pethidine hydrochloride (0·5 mg./ml. in 5 per cent. dextrose in water) was dripped continually or intermittently by vein as required. The principal types of operation undertaken were plastic, orthopædic and other extra-abdominal procedures not requiring deep anæsthesia. In the total patients operated on, the thiopentone required was 675 mg./hour in those receiving thiopentone and nitrous oxide alone, whereas only 350 mg./hour was required in those receiving the pethidine drip. In the pethidine series therefore 48·1 per cent. thiopentone was required. In patients whose operative procedures exceeded 4 hours the respective thiopentone requirements per hour were 350 mg. and 247 mg., or a decrease in thiopentone requirement of 29·4 per cent. In those undergoing plastic procedures the figures were 596 mg. and 380 mg./hour respectively, or a decrease in thiopentone requirement of 36·2 per cent. An average of 72·3 mg./hour of pethidine was required as reinforcement to the analgesia provided by nitrous oxide. The authors conclude

that the continuous drip method of administration of pethidine gives a smoother anæsthesia, with less danger of severe respiratory depression and hypotension than is obtained by single injections of pethidine, and that the technique is a valuable addition to thiopentone sodium—nitrous oxide—oxygen anæsthesia. Details are given of investigations to determine the effect of the technique on tidal volume, minute volume of respiration, oxygen utilisation and alterations in pH in carbon dioxide tension in the arterial blood. S. L. W.

Reserpine in Delirium Tremens. M. Avol and P. J. Vogel. (*J. Amer. med. Ass.*, 1955, **159**, 1516.) Twenty-three patients suffering from delirum tremens were treated by intramuscular or intravenous injections of reserpine. The patients were free of their symptoms of acute hallucinations in an average of 18 hours; most of them were free of symptoms within 24 hours. The shortest time noted was 9 hours (in 2 patients) and the longest 48 hours (in 2 patients). The drug was given in a dose of 2·5 mg. intravenously or intramuscularly followed by a similar dose in 3 or 4 hours if the patients were still agitated. Ordinarily the patients would begin to quieten down in 6 or 8 hours. Many patients were given a dose of paraldehyde (10 ml.) initially in order to keep them controlled for the first few hours. All patients were fully awake or only slightly drowsy the following day. Despite the relatively large doses used no ill-effects were noted in this series. Hypertensive cardiovascular disease *per se* is not a contraindication to the use of the drug, though the drug should be used with caution in these instances. The use of reserpine greatly shortened the time required to free these patients of their post-alcoholic withdrawal symptoms and made them much easier to nurse.

S. L. W.

Zoxazolamine, Clinical Experience with. W. Amols. (*J. Amer. med. Ass.*, 1956, **160**, 742). Electromyography was used to provide a crude but objective index of the effect of relaxant drugs and was found to be a useful supplement to clinical evaluation. Arbitrary values are assigned to certain parts of the tracing according to the amount of electrical activity recorded when the limb is in a position requiring no voluntary effort, and the extent of muscular relaxation obtained by a drug and its duration are shown by plotting degrees of spontaneous relaxation against time. Premedication with chlorpromazine was found to potentiate the effect of mephenesin and it was therefore tried in conjunction with zoxazolamine (Flexin), a muscle relaxant believed to act in the same way as mephenesin by depressing polysynaptic pathways in the central nervous system. Zoxazolamine alone, in doses of 500 to 1500 mg. by mouth four times a day, gives only slight relaxation of spastic muscles. After premedication with chlorpromazine in doses of 10 to 50 mg. by mouth a significant degree of relaxation is obtained with 500 mg. of zoxazolamine four times daily in patients with spastic extremities. No effect was observed in patients with decerebrate rigidity, torticollis, or disease of the basal ganglions. Two patients with grand mal seizures whose anticonvulsant therapy was supplemented by zoxazolamine were made worse. 13 out of 16 patients with spastic extremities secondary to spinal cord disease showed a definite reduction in muscle tone. The amount of relaxation varied greatly in different patients and in the same patient at different times, but the duration of the effect was relatively constant at 6 hours. All patients whose spastic extremities were affected favourably by the treatment complained of greater weakness in the extremities and most of those showing the best response gave up the treatment for this reason. The principal role of muscle-relaxant therapy is as an adjunct to nursing care and physical therapy, to alleviate the discomfort and inconvenience of the spasticity. H. T. B.

BACTERIOLOGY

Tetrazolium Reduction as a means of Differentiating *Streptococcus fæcalis* from *Streptococcus fæcium*. E. M. Barnes. (*J. gen. Microbiol.*, 1956, **14**, 57.) The author reports studies on the reduction of 2:3:5-triphenyltetrazolium chloride (tetrazolium) in a glucose nutrient medium by the Lancefield group D streptococci. Tetrazolium is colourless in the oxidised form and is reduced to the insoluble red triphenylformazan. It was found that ability to reduce this compound served to distinguish strains of *Streptococcus fæcalis* from strains of *Str. fæcium* (Orla-Jensen). Differences in tetrazolium reduction agreed with other tests used to separate the species. Tetrazolium reduction in a 24 hour growth of the test organism in tetrazolium-glucose medium was determined by extracting the formazan with *n*-butanol and estimating colorimetrically. Percentage reduction was calculated by reference to a standard curve, which was obtained from complete reduction of solutions of known tetrazolium content on treatment with ascorbic acid in alkaline solution. 68 strains of *Str. fæcium* and 34 strains of *Str. fæcalis* were tested in the medium (pH 6·8–7·0). Only 6 strains of *S. fæcium* gave over 50 per cent. reduction of tetrazolium, whereas only 2 strains of *Str. fæcalis* gave less than 50 per cent. reduction. Thus the difference in reducing powers of the two species was characteristic. Maximum differentiation is achieved if the test is carried out at pH 6·0–6·2, but the formation of a colourless reduction product in addition to the formazan under these conditions make the use of a less acid medium necessary for quantitative determinations. No differentiation could be achieved in a tryptic digest medium containing tetrazolium. Rapid qualitative methods for differentiating the two species, using either solid or liquid media (pH 6·0–6·1) are described. The author considers that within the Lancefield group D, *Str. fæcium* appears to occupy an intermediate position between *Str. fæcalis* on the one hand and *Str. durans* and *Str. bovis* on the other. B. A. W.

Viable Bacteria in a Culture, Method for Determining the Proportion of. E. O. Powell. (*J. gen. Microbiol.*, 1956, **14**, 153). A rapid method of determining proportions of viable organisms by counts on cellophane graticules is described. The author gives details of a special engraving tool, in which a pile of razor blades, conveniently 11 in number, are held in a block. Discs of cellophane are pressed twice against the blade edges, one impression being made at right angles to the other. A graticule can be made in about 1 minute, including punching out the disc. Suitable cellophane for use is specified and methods of cleaning are described. In making a count, the disc is placed on the surface of a nutrient agar plate, preferably previously dried, and the centre of the disc inoculated with one loopful of a suitable dilution of the culture. A stream of air is blown over the surface to hasten drying, and then the numbers of single organisms and of groups in each square is noted. After 2–6 hours incubation, the number of groups in each square is again counted, the difference in the group count being taken as the number of viable organisms among the single organisms originally counted. Although only a limited range of organisms had been used with this method, the results were found reproducible and agreed generally with those obtained by conventional procedures. The chief advantages are speed, directness, absence of lengthy dilutions, avoidance of errors due to clumping and ability to count organisms which give spreading or diffuse colonies. The principal disadvantages appear to be limited statistical accuracy, apparent non-viability of organisms with a long lag phase and personal difficulties in assessment. B. A. W.