THE ANALYST

A Monthly Publication dealing with all branches of Analytical Chemistry: the Journal of the Society of Public Analysts and Other Analytical Chemists

Editor: F. L. OKELL, F.R.I.C.
7-8, IDOL LANE, LONDON, E.C.3
Telephone: MANsion House 6608

Published for the Society by
W. HEFFER & SONS, LTD., CAMBRIDGE, ENGLAND

Volume 78 Price 6s. 6d.
Subscription Rate, inclusive of Abstracts C, 80/- per annum Post Free
No. 929, Pages 449-508 August, 1953

IMPORTANT NOTICE TO SUBSCRIBERS

(Other than Members of the Society)

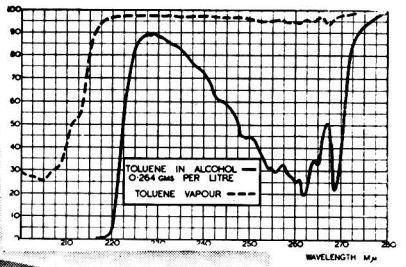
All Subscriptions and renewals to the Journal, including Abstracts C., should be sent through a Bookseller or direct to

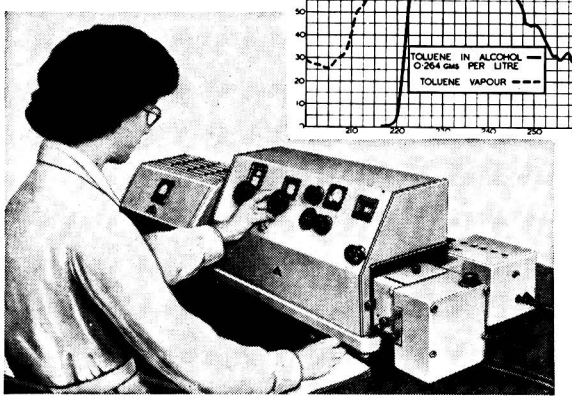
W. HEFFER & SONS LTD, CAMBRIDGE, ENGLAND.

Price 6/6, postage 2d.; or to Subscribers in advance post free £4 per annum

N.B.—Members send their subscriptions to the Hon. Treasurer

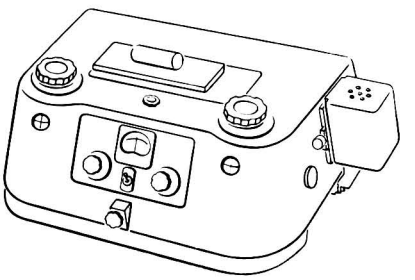
UNICAM
SPECTROPHOTOMETERS





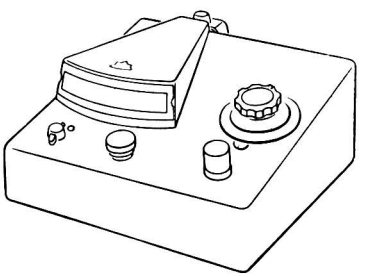
SP.500

The well-known Unicam Quartz Spectrophotometer makes possible analysis by light absorption measurement at all wavelengths from 2,000Å to 10,000Å. The quality of its performance and simplicity of operation combine to make the instrument ideally suitable for research, process control or routine determinations.



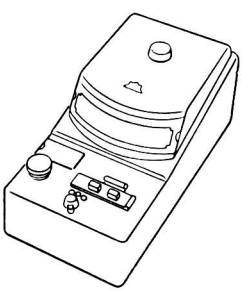
SP.600

This new spectrophotometer offers at moderate price the many advantages of up-to-date analytical methods. The high resolution of the glass prism monochromator combined with a reliable electronic system gives exceptional performance in the visible range (3,600—10,000Å).



SP.350

Chemists in clinical or biochemical laboratories appreciate the diffraction grating fitted in this spectrophotometer which allows them to follow an end point in a changing system. A use will be found for this reliable instrument in almost every laboratory.



SP.300

The demand for a simple, robust filter-type colorimeter giving a consistently high performance over long periods is amply met by the Unicam SP.300 Colorimeter. New applications are continually being found for this reliable, inexpensive instrument that is strongly built and easy to operate.

Illustrated leaflets describing any of these instruments in detail will gladly be sent on request.

UNICAM INSTRUMENTS (CAMBRIDGE) LTD · ARBURY WORKS · CAMBRIDGE
U125 JNRTX

THE ANALYST

EDITORIAL

DRUMMOND MEMORIAL FUND

A COMMITTEE has been formed under the Chairmanship of Lord Woolton with the object of endowing a Drummond Research Fellowship in Nutrition as a memorial to the late Sir Jack Drummond, who was an active member of our Society for thirty years.

The cost of the Fellowship is estimated at £25,000, and it is proposed that the fund should be administered by a body of University Trustees and be tenable in any university or appropriate research institution.

Lord Woolton, in a letter dated July 11th, 1953, to *The Times*, wrote—

"The anniversary of the tragic murder of Sir Jack Drummond and his family whilst on holiday last year recalls the great debt that we in Great Britain owe to him for his work as Scientific Adviser to the Ministry of Food during the war.

Drummond was peculiarly fitted for this task. He had devoted twenty years to work in the field of experimental nutrition and the nation had begun to realise the immense importance of food in relation to health and the prevention of disease. This was in no small part due to his book "The Englishman's Food," a profound study of the effects of changes in the food habits of our people in the previous five hundred years.

During the 1939 war Drummond used this knowledge admirably in the interests of the nation. He applied himself with almost a crusading zeal to meeting the particular nutritional needs of the growing child, the expectant mother and nursing mother, and the sick. As a result we introduced, through the Ministry of Food, important measures that have had far-reaching consequences and have left their permanent mark on the social structure of our country by contributing to the health and well-being of our young people.

This work ought not to be forgotten, and a committee has been formed, under my chairmanship, to ensure that his name and work may live. . . . We beg that all who share our sense of indebtedness to the work of Drummond . . . will subscribe generously to this appeal.

The members of the committee who join me in this appeal are Lord Horder and Professor E. C. Dodds representing medicine, Sir Harold Himsworth and Sir William Slater, secretaries of the Medical and Agricultural Research Councils, respectively, and Dr. Norman Wright of the Ministry of Food. Academic biochemistry is represented by Professors A. C. Chibnall, G. F. Marrian and F. G. Young, while the Provost of University College, Dr. Ifor Evans, is also a member. Industry is represented by Mr. Leonard Anderson, Dr. H. J. Channon, Sir Harry Jephcott and Mr. Wilfred Vernon.

Subscriptions, whether by gift or covenant, should be addressed to The Drummond Memorial Fund, c/o The Westminster Bank Ltd., 154 Harley Street, London, W.1."

Those who have benefited professionally, or commercially, by the work of Sir Jack Drummond will need no persuasion to contribute to a fund for the continuance of his life's work; others may well wish to do so by way of thanks for his contributions to the feeding of a nation at war.

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

THE FARADAY SOCIETY

THE following letters were exchanged between the President, Dr. D. W. Kent-Jones, and the President of the Faraday Society on the occasion of the 50th Anniversary of the foundation of the Faraday Society, celebrated on April 16th, 1953.

To the President of the Faraday Society

Dear Sir,

The President, Officers and Council, and the Members of the Society of Public Analysts and Other Analytical Chemists express their cordial congratulations and good wishes to the Faraday Society on the occasion of the celebration of the Jubilee of your Society's foundation.

For many years my Society has watched with interest and great admiration the activity of your Society in promoting the study of Physical Chemistry and Metallography, Electrometallurgy, and Pure and Applied Electrochemistry; and we have been particularly interested in the discussions and symposia that you have promoted on the many subjects whose full consideration is outside the province of the specialised scientific societies, and which require the bringing together of men engaged in widely varying aspects of Science. Especially we remember with gratitude your Society's work in emphasising the need for the co-ordination of science with practice.

In conveying to you our congratulations for the success that has attended your Society during the past fifty years we also extend to your Society our continued good wishes for its future.

Yours faithfully,

(*Signed*) D. W. KENT-JONES,

President.

To the President of the Society of Public Analysts and Other Analytical Chemists

Dear Mr. President,

On behalf of the Council and Members of the Faraday Society may I express our appreciation of the cordial congratulations and good wishes of your Society on the occasion of our Golden Jubilee.

We thank you also for your tribute to the work we do in bringing together scientists for discussions in areas which lie beyond the range of specialised scientific societies, and we look forward to a continued future of service for science in these paths.

Very sincerely yours,

(*Signed*) HUGH S. TAYLOR,

President.

THE BERNARD DYER MEMORIAL MEDAL

THE Deputy Master and Comptroller of the Royal Mint, Sir L. Lionel H. Thompson, has selected the Bernard Dyer Memorial Medal as one of the examples of contemporary medallic work in this country to be submitted for an exhibition, opening on October 5th, 1953, at the Palazzo Venezia, Rome, organised by the Fédération Internationale Des Editeurs de Médailles.

The Medal was also exhibited by the Royal Mint at the Summer Exhibition of the Royal Academy at Burlington House this year.

The Efficient Planning of Microbiological Assays

Illustrated by Assays of Cobalamin

By ERIC C. WOOD

(*Presented at the meeting of the Society on Wednesday, November 5th*, 1952)

The limitations of the design usually employed for microbiological assays are discussed, with a series of assays of cobalamin as example. Transformation of dose and response to their logarithms is shown to give a linear relationship between the transformed variables. A more efficient design for such assays can be based on this fact; it readily enables estimates to be made not only of the potency of a test preparation, but also of that potency's fiducial limits and of the validity of the assay. In a statistical appendix, concise formulae are given for calculating these quantities for the four most likely forms of the general design put forward.

In the main, published microbiological assay methods all follow the same basic principles in their design. There should be omitted from this generalisation those assays—notably of riboflavin and of nicotinic acid—in which the relation between dose and response is sensibly linear over a reasonable range, because for such "slope-ratio" assays the most efficient designs have been fairly thoroughly worked out[1,2,3]; the Analytical Methods Committee's Report[4] on the microbiological assay of these two vitamins embodies the improved designs. But most assays of vitamins and amino-acids show a curvilinear relation between dose and response and are thus not amenable to the same treatment. Many of these assays have been shown[5] to give a linear relation between the logarithm of the dose and the logarithm of the response (referred to hereafter for the sake of brevity as log-dose and log-response, respectively) and the implications for the design of the assay and the calculation of the result have been briefly indicated. Nevertheless, most papers dealing with such assays still put forward what might be called the "common-sense" type of design. The protocols of half a dozen recent assays of cobalamin following this same design form the basis of the discussions and suggestions below; but these are, in general, applicable to all assays linearised by the same transformations.

The "common-sense" design and its limitations

The technique used in these assays may be briefly described. Seven or eight graduated doses of pure cobalamin are each put into three or four test tubes of basal medium to provide data for plotting the "standard curve" of response against dose—the response being in this instance the turbidity caused by the growth of *Lactobacillus leichmannii*; a similar number of tubes at the zero-dose level (the "blank") are also included. Graduated doses of the test preparation are put into other tubes at three or four levels. This type of design follows naturally from the original research that led to the establishment of the assay technique; the primary need was to find an organism whose growth was related to the amount of cobalamin present in its environment and also to find the conditions under which reproducible and specific results could be obtained. This would entail a careful and intensive investigation into the shape and nature of the dose - response curve over as wide a range of doses and on as many occasions as possible. When an assay of the cobalamin content of some foodstuff was required, the use of a further series of tubes at a few dose-levels of the test preparation followed naturally. Then, by applying the mean response at each sample dose-level to the standard curve, the corresponding vitamin content could be read off and the assay result worked out. Fig. 1 illustrates a typical assay of this kind, with seven levels of standard besides the "blank," and three levels of sample. As previously stated, the design of this assay is also followed in many published papers dealing with the assay of a wide variety of vitamins and amino-acids. This particular example is, of its kind, well-conducted and satisfactory.

There is one aspect of this design—its wastefulness—that demands immediate consideration. If part of any experiment could have been omitted without the slightest effect on the

conclusions from it, then the experiment has been to that extent conducted inefficiently. A glance at the figure will show that at least the "blank" and the lowest and highest doses of standard preparation are superfluous; they contribute nothing to determining the shape of that part of the standard preparation curve from which the potency of the sample is estimated. Even if the test preparation responses had covered a wider range, the top part of the curve is too nearly horizontal for reasonably precise use; a small variation in response

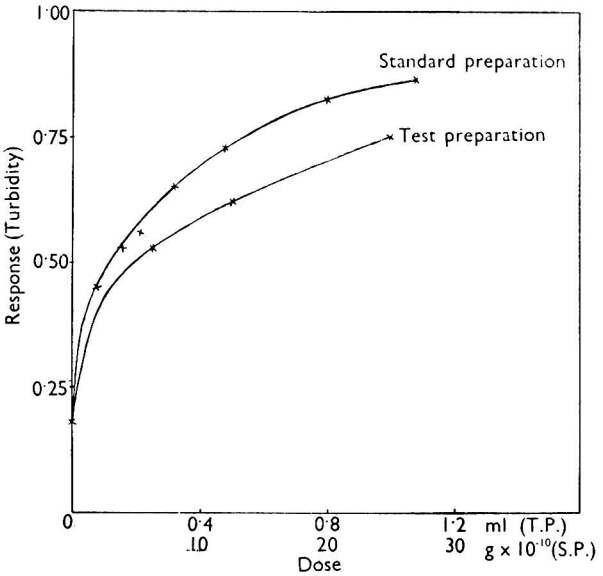


Fig. 1. A typical pair of dose - response curves in a microbiological assay of cobalamin. The response is the turbidity produced by the growth of *L. leichmannii*

corresponds to far too large a difference in dose. The bottom end of the curve is also undesirable for normal use, as the doses and responses are too small for precise measurement (but see below). If, then, the central portion only of the dose - response curve is to be used, there is no point in using dose-levels outside that range.

It may be that the analyst likes to include these doses, even though the responses to them are not used in calculating the result of the assay, because the fact that these responses are in accordance with expectation gives him an added feeling of confidence that all has gone well. It must be pointed out, however, that even had they *not* been in accordance with expectation, this could not reasonably be allowed to affect the computation of the assay, or even one's confidence in it, if all the responses actually used in the computation were in every way normal. If this be admitted, the only argument for including these superfluous dose-levels falls to the ground.

There is an analogy here to macrobiological assays in which, say, rats are dosed with various amounts of vitamin A and their weight increases measured. Doses below a certain minimum are not used, because the rats receiving them would merely lose weight and die, and doses above a certain maximum are likewise omitted, because on them all the animals would grow at rates approaching the optimum and discrimination would be poor. All the doses given, whether of standard preparation or test preparation, are intended to be on the central part of the dose - response curve, the part of maximum usefulness. A clear distinction must be made between the needs of the research worker, investigating the nature of the response of the organism to the vitamin or other nutrient, and those of the analyst for whom the response is no more than a means to an end and who is interested only in the amount of the vitamin present in some test preparation. The experimental designs used by these two kinds of experimenter can be as completely different as the purposes they are intended to serve.

Another reason why the design illustrated in Fig. 1 is inefficient is that there are too

many standard preparation doses in relation to the number of test preparation doses. They may have been used to ensure, in the absence of knowledge of the potency of the sample, that the whole possible range of test preparation responses is covered by the standard curve. This approach to the problem is wrong, for we have seen that only responses falling on the central part of the curve should be used in calculating the result. A more correct solution is to use more test than standard preparation doses—the less the analyst's knowledge of the expected potency of the sample, the more doses there will have to be—and to reject from the calculations those test preparation responses outside the useful range. These observations are wasted, certainly, but this is necessary waste—the price paid for being unable to predict the approximate test preparation potency; indeed, if a shrewd guess can be made, there may be little or no waste. In the present instance, for example, the sample doses were well chosen and no observation was wasted; but four levels of standard preparation would have been enough to cover the working part of the curve. With four tubes at each level, twenty-eight tubes would have been used in this assay instead of forty-four, representing a real saving of materials, of space in the incubator and of time—and all without any loss of information. The difference is not so marked when two or more test preparations are assayed simultaneously, but there is still a useful measure of economy.

Hence it is well worth while, even if nothing further is done, to reduce the number of standard preparation dose-levels to cover only the working part of the curve and to equalise or nearly to equalise the numbers of standard preparation and test preparation dose-levels over this range. The discarded observations are of no use to the analyst; he should retain only those that can contribute to the final result.

Precision, accuracy and validity

Another fault of the design illustrated by Fig. 1, coupled with the "direct reading" method of obtaining the result of the assay, is that it is so difficult to estimate from it the precision of the result that, in practice, it is hardly ever computed. In consequence, if a food is assayed in two different laboratories, or by two different techniques, it is not possible to say whether the difference between the results is significant or may reasonably be attributed to experimental error. To attempt to compare the results of two assays without knowledge of the precision of the quantities compared is like measuring a distance without a unit of length. In the direct-reading method, incidentally, the agreement between the potencies of the test preparation, as read at the three different dose-levels, is no guide whatever to the precision of the mean result; the three estimates might be all exactly the same and yet the result might be seriously lacking in precision.

Even the accuracy of the result as well as its precision may be in doubt. It is usual to take the arithmetical average of the three potencies read from the curve; yet the slope of the standard preparation curve varies greatly from the lowest to the highest response, and—other things being equal—the more nearly vertical the standard preparation curve is, the greater the weight to be attached to a potency read from it. This suggests that an assay of this kind would be improved by working only near the bottom end of the curve, and there are other advantages to be gained, to some of which Pritchard has drawn attention.[6] Such a procedure is impracticable, however, unless means can be devised to improve the precision of measuring both the small doses required and the correspondingly small responses. The fact remains that the average of the three directly-read estimates is probably not the best final estimate. Moreover, the freehand drawing of the standard preparation curve introduces a subjective element into the result, and this also is objectionable.

Related to these considerations is the lack of a reliable check on the validity of the assay—that is, on the absence from the test preparation of interfering substances that may have modified the response of the test organism to the factor being measured. One criterion of invalidity often advocated by users of the "direct-reading" technique is the presence of "drift"—a regular trend upwards or downwards—in the potencies as read from the curve at the various dose-levels. It is often stated that the three or four estimates should agree within 10 per cent. among themselves and should not be in order of magnitude. This is not reliable and is too arbitrary; whether 10 per cent. is a severe or a lax criterion depends, among other factors, on the distance apart of the three dose-levels used. This is not to say that the test is not useful; it certainly is, particularly in the hands of an experienced worker. But a more objective and consistent test is needed.

THE REMEDY—THE "LOG-LOG" TRANSFORMATION

Proper statistical treatment of the assay results will remove all these objections and provide the best estimate not only of the mean result, but also of its precision and of the validity of the assay. Such treatment, however, is difficult or even impossible so long as knowledge of the dose - response relationship is confined to a freehand curve. An algebraic equation connecting the dose with the response is required, and if possible it should demonstrate the existence of functions of the dose and the response that are linearly related to each other, for then the arithmetic is much simplified and the calculations become standardised.

When the dose is not linearly related to the response itself, logarithms are usually next tried. Most bio-assays on animals such as rats and rabbits give, over a certain range, a sensibly linear relation between log-dose and response. The cobalamin assays now being considered do not behave in this way; the curve obtained is perhaps easier to draw freehand than the original curve, but still has no linear part. Because of previous findings with amino-acid assays,[5] I tried plotting log-response against log-dose. This linearised all the six assays examined, although the range of the linear relation differs somewhat from assay to assay. The graphs of the six standard preparation curves are shown in Fig. 2.* The

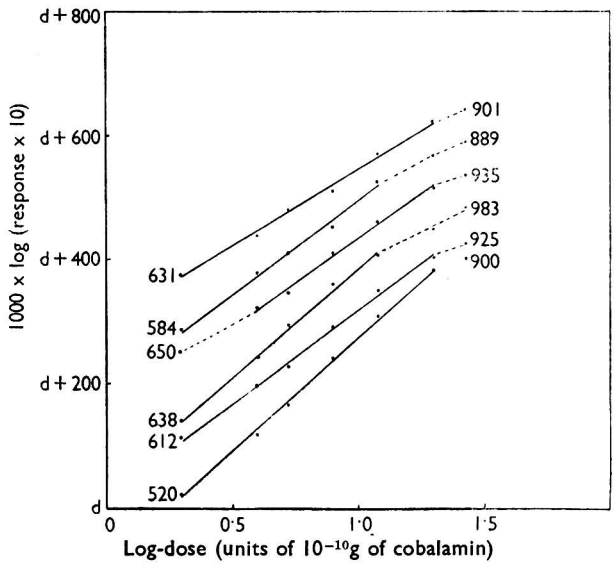


Fig. 2. Log-dose plotted against log-response for six consecutive S.P. curves of the type shown in Fig. 1. To avoid confusion, the true zero of the ordinate axis varies from line to line; the numerical values of the highest and lowest log-responses for each line are indicated on the graph

variations from assay to assay in general level of response, in slope and in range of linear portion are in accordance with all experience of bio-assay and reflect the inherent variability of living organisms. On the evidence of these six assays, however, log-dose is linearly related to log-response over a range of log-dose from 0·6 to 1·2 approximately. Transformation of the assay of Fig. 1 gave the result shown in Fig. 3; here the linear part of the standard preparation curve is from log-dose 0·3 to 1·3 at least. Moreover, the points for the test preparation also lie on a straight line, within the same range of log-response as the linear part of the line for the standard preparation, the two lines being parallel or nearly so. This forms a satisfactory basis for statistical treatment, although the necessary computations are rather lengthy, because the design does not lend itself to concise formulae; the unsymmetrical pattern as between standard and sample and the unequal spacing of the standard

* For convenience, the response metameter used is 1000 × log (response × 10), so that, *e.g.*, a response of 0·331 becomes 1000 × log 3·31 = 520. To avoid the six lines being confusingly close together and even crossing, the zero of the vertical axis is not the same for every line.

preparation doses complicate the calculations. Nevertheless, they can be readily made. The best estimate of potency of the test preparation is found to be 0·0295 μg per g, with 95 per cent. fiducial limits of 0·0269 to 0·0323 μg per g; there is no evidence that the assay is in any way invalid; there is no significant departure from linearity between standard preparation log-dose limits of at least 0·6 and 1·3; and the standard error of a single observation is 14·4 units of the response metameter. The assay is thus a reasonably precise one, as microbiological assays go, with fiducial limits slightly less than 10 per cent. either side of the mean result.

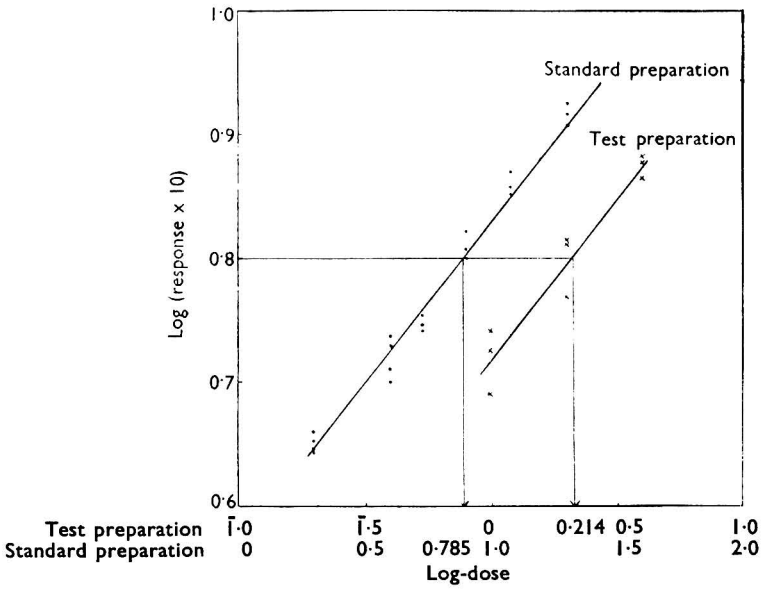


Fig. 3. The data of Fig. 1 plotted between the limits of log-response from 0·6 to 0·95 after the "log-log" transformation. Each point plotted is an individual log-response

A MORE EFFICIENT DESIGN

This, however, does not end the matter. The fact that by means of the log-log transformation the maximum of information can be extracted from the results of this particular assay does not mean that anyone would wish to go on carrying out assays of the same design and working out the results in the same way. The computations are too involved, and much of the experimental labour of the assay is wasted. Once it is known that the log-log transformation is applicable to a certain type of assay, well-known principles can be used to define the most efficient experimental design and to provide concise formulae from which the assay result, its fiducial limits and other necessary information can be rapidly and conveniently extracted.

The conditions to be complied with are four in number. First, for both the standard and the test preparations, the log-doses should be equally spaced, *i.e.*, the doses themselves should be in geometrical progression. It is conducive to simple formulae if the spacing of the test preparation doses is the same as that of the standard preparation doses. Arithmetically, however, what the actual spacing is matters little. Secondly, the lowest and highest standard preparation doses should be so chosen as to include most of the known linear part of the log-log line without risk of going outside it. This clearly means some degree of compromise; for the present series of assays, a range of log-doses from 0·6 or 0·7 to 1·1 or 1·2 appears reasonable. Thirdly, the number of standard preparation doses should be three or four. More can be used, but then the amount of experimental work and of arithmetic both increase with little, if any, compensating advantage. There is not much to

choose between the three-dose and four-dose designs, and the latter may commend itself to those who, having previously used seven or eight doses, feel uneasy at too drastic a reduction in number. Moreover, if four doses are used and it is afterwards found that the highest or the lowest dose is off the linear part of the curve, the responses at this level can be discarded and the remaining three dose-levels used in the calculations. In the present instance, the log-doses would be then, say, 0·6, 0·8, 1·0 and 1·2 for a wide spacing, or 0·7, 0·85, 1·0 and 1·15 for a narrower one. Lastly, the number of test preparation doses should be so chosen that the experimenter is reasonably sure of finding that at least two, and preferably the same number as there are standard preparation doses, give mean responses within the range of the standard preparation doses or only just outside. If the approximate potency of the test preparation can be shrewdly guessed, the number of test preparation doses can be made equal to the number of standard preparation doses; if not, it may be necessary to use one or even two extra dose-levels of sample and be prepared to discard the results from the highest or lowest level when computing the result of the assay.

An assay of this design should nearly always give three or four standard preparation dose-levels on the linear part of the log-log graph, with an equal number of test preparation dose-levels, or perhaps one less, in the same range. The formulae given in the Appendix are then applicable and will be found to lend themselves to reasonably rapid calculation. The only part of the computations that is at all lengthy is the estimation of the standard error, s, if this is found from the differences between log-responses in each dose-group by the well-known procedure involving squaring, adding and so on. There is, however, a permissible short-cut, unless exact fiducial limits are essential. The standard error can be estimated from the range of log-responses at each dose-level. Details of both methods and a comparison between them have been published elsewhere,[7] but the range method may be demonstrated by applying it to the results of the assay illustrated in Figs. 1 and 3, as follows.

The ranges of the standard preparation log-responses at each level used in the calculations are 37, 12, 21, 18, 18, and of the test preparation responses, 50, 46, 18. The mean range is 27·5. On dividing by the factor 2·06* the standard error becomes 13·4. The estimate obtained by the "orthodox" process is, as previously stated, 14·4. The difference in the fiducial limits brought about by using this quick but rather unprecise method of estimating the standard error is small; they are calculated as 0·0271 to 0·0320 instead of 0·0269 to 0·0323 μg per g.

In conclusion, two points deserve emphasis. First, it is not suggested that because the design described above lends itself to the ready calculation not only of the mean result, but also of other valuable statistics, the full sequence of calculations should be traversed on every assay. The results of the assay should always first be plotted on graph paper, after applying the log-log transformation, and it is then usually easy to see over what ranges the standard preparation and test preparation mean responses are linear and if the lines are reasonably parallel. If inspection shows that all is well for three or four doses of each preparation, the appropriate formula for M, the mean log-potency, can be selected from the Statistical Appendix and the mean result calculated—a process taking no longer than any other way of working out the result and giving an objective and unimpeachable estimate. If upon occasion further information is required, or if there is any reason to doubt the soundness of the assay, the data lend themselves to proper statistical computation, with a minimum of time and trouble, on the lines set out in the Appendix.

Secondly, the designs described and the computations arising therefrom are appropriate only when the assay is such as to give a linear relationship over a reasonable range between log-dose and log-response (or some other function of the response). They must not be applied to some assay in which the dose - response relationship is of an entirely different type. But in those assays to which they are applicable—and in my experience that means a considerable number of vitamin and amino-acid assays—these designs have many advantages, not the least of which is the reduction they afford in the amount of work required for a given amount of information.

* The factor by which the mean range must be divided to estimate the standard error is 2·06 only when there are 4 observations in each dose group. For sets of 3 observations the factor is 1·69; for sets of 5, 2·33, and for sets of 6, 2·53.

Statistical Appendix

The following notes on the concise computations applicable to certain experimental designs are not meant to be a guide to the statistical theory of biological assay; for that, reference must be made to a suitable treatise, such as Finney's recently published book.[8] Therein will be found the full explanation of the various steps in the computations and the theoretical meaning of the various quantities calculated.

It is assumed that n observations have been made at each dose-level of standard and sample. The lowest dose is taken as the unit, so that the potency-ratio, R, is that of the lowest dose of test preparation to the lowest dose of standard preparation. The doses are in geometrical progression with multiplier D, so that the log-doses are in arithmetical progression and spaced by an interval $d = \log D$. The doses of each preparation are thus 1, D, D,^{2} and so on, and the corresponding log-doses are 0, d, $2d$, and so on. Reference to a (k, k') assay means an assay in which k levels of standard and k' levels of sample are used in the *calculations* (more may have been included in the original design but discarded for some good reason). The standard error of a single observation, s, is assumed to have been either calculated from the sums of the squares of the log-responses at each dose-level or estimated from their mean range as explained in the text. Each quantity y refers to the *sum* of the n log-responses at each dose-level; once s has been calculated, the rest of the calculations are made from these sums, the individual log-responses not being used again.

It is also assumed that the quantity $g = t^2s^2V(b)/b^2$ would be found, if calculated, to be small enough for the fiducial limits of the mean result to be computed without material error by regarding $(1 - g)$ as equal to unity.* In all those microbiological assays I have examined in which the agreement between replicate observations is reasonably good and the linearity after log-log transformation is satisfactory, g is always so small that the assumption is fully justified; for g to be negligible it should be less than 0·1, whereas in these microbiological assays it is usually of the order of 0·01. Strictly speaking, it should be calculated for each assay, but unless the assay shows obvious signs of lack of precision, such as would lead the experienced worker to view it with grave suspicion in any event, this should be unnecessary.

The (3, 3) assay design—

Log-doses	(S.P.)—0	d	$2d$	(T.P.)—0	d	$2d$
Sum of n log-responses ..	y_1	y_2	y_3	y_4	y_5	y_6

The first step is to calculate a series of quantities L_p, L_1, and so on, by multiplying each y by the "detached coefficient" given below it in Table I and adding the results. For example, $L_p = -y_1 - y_2 - y_3 + y_4 + y_5 + y_6$. The standard error of each such quantity is also given in Table I.

Table I

Detached coefficients for (3, 3) assay

	y_1	y_2	y_3	y_4	y_5	y_6	Standard error
L_p	−1	−1	−1	+1	+1	+1	$s\sqrt{6n}$
L_1	−1	0	+1	−1	0	+1	$s\sqrt{4n}$
L'_1	−1	0	+1	+1	0	−1	$s\sqrt{4n}$
L_2	+1	−2	+1	+1	−2	+1	$s\sqrt{12n}$
L'_2	+1	−2	+1	−1	+2	−1	$s\sqrt{12n}$

In a table of the statistical quantity t (see almost any book on statistics) look up its value for $6(n - 1)$ degrees of freedom and $P = 0{\cdot}05$ (usually). For $n = 3$, 4 or 5, $t = 2{\cdot}18$, 2·10 or 2·06, respectively. The ratio of each of the above quantities L to its standard error should now be compared with t, *i.e.*, its significance should be tested.† The interpretation of the results of these tests is as follows—

L_p measures the extent to which the mean response to the test preparation differs from the mean response to the standard preparation. It should be small, preferably

* The quantity b is the mean slope of the two log-dose - log-response lines and $V(b)$ is its variance.

† When s is estimated from the mean range, the use of t is not, strictly speaking, correct; tables of the correct quantity to use in its place have been given by Lord.[9] In microbiological assays in which g is of the order of 0·01, however, the error introduced by using t is negligible.

not significant; if significant, the assay may still be sound, provided that it is satisfactory in all other respects, although it may not be very precise.

L_1 measures the mean slope of the assay; it should be highly significant—the higher, the better.

The other three quantities measure different kinds of departure from linearity or parallelism. They must all be without significance; the significance of one or more would show that the assay was for some reason invalid.

With a little experience these tests of significance may become partly or wholly unnecessary. Inspection of the graph of the assay results and of the numerical values of L_p, L_1, etc., will often show that all is well and that no actual tests are needed.

If the assay appears to be sound, calculate the following quantities in turn—

(*i*) $C = 4d/3L_1$.

(*ii*) The mean log-potency $M = C.L_p$; its antilogarithm, R, is the mean potency-ratio.

(*iii*) If the 95 per cent. fiducial limits are required, calculate $Q = ts\sqrt{6n(1 + 2L_p^2/L_1^2)}$. Then M_L and $M_U = C(L_p \pm Q)$, and the corresponding antilogarithms are the fiducial limits sought.

THE (4, 4) ASSAY DESIGN—

As before, a series of quantities is calculated by the method of detached coefficients (see Table II) from the log-responses at each dose-level, from the following values—

Log-dose	(S.P.)—0	d	$2d$	$3d$	(T.P.)—0	d	$2d$	$3d$
Sum of n log-responses	y_1	y_2	y_3	y_4	y_5	y_6	y_7	y_8

TABLE II

DETACHED COEFFICIENTS FOR (4, 4) ASSAY

	y_1	y_2	y_3	y_4	y_5	y_6	y_7	y_8	Standard error
L_p	−1	−1	−1	−1	+1	+1	+1	+1	$s\sqrt{8n}$
L_1	−3	−1	+1	+3	−3	−1	+1	+3	$s\sqrt{40n}$
L'_1	+3	+1	−1	−3	−3	−1	+1	+3	$s\sqrt{40n}$
L_2	+1	−1	−1	+1	+1	−1	−1	+1	$s\sqrt{8n}$
L'_2	−1	+1	+1	−1	+1	−1	−1	+1	$s\sqrt{8n}$
L_3	−1	+3	−3	+1	−1	+3	−3	+1	$s\sqrt{40n}$
L'_3	+1	−3	+3	−1	−1	+3	−3	−1	$s\sqrt{40n}$

The significance of these quantities must be examined as before, t having now $8(n - 1)$ degrees of freedom; for $n = 3$, 4 or 5, $t = 2{\cdot}12$, 2·06 or 2·04, respectively. L_p and L_1 have the same meaning in all assay designs, and all the others measure one kind or another of departure from the ideal condition of two parallel straight lines. The remarks made under the heading of the (3, 3) design apply throughout.

If all is well, $C = 5d/L_1$, $M = C.L_p$ and $R =$ antilog M as before.

The fiducial limits of the log-potency ratio, M_L and M_U, are given by $C(L_p \pm Q)$, where $Q = ts\sqrt{8n(1 + 5L^2_p/L^2_1)}$. The fiducial limits of R, the potency-ratio, are the antilogarithms of M_L and M_U.

THE (3, 2) ASSAY DESIGN—

If the sum of the n log-responses at each dose-level is given by (S.P.) y_1, y_2, y_3, (T.P.) y_4, y_5, the table of detached coefficients is as shown in Table III.

TABLE III

DETACHED COEFFICIENTS FOR (3, 2) ASSAY

	y_1	y_2	y_3	y_4	y_5	Standard error
L_p	−2	−2	−2	+3	+3	$s\sqrt{30n}$
L_1	−2	0	+2	−1	+1	$s\sqrt{10n}$
L'_1	+1	0	−1	−2	+2	$s\sqrt{10n}$
L_2	+1	−2	+1	0	0	$s\sqrt{6n}$

In testing the significance of these quantities, t has $5(n-1)$ degrees of freedom; for $n = 3$, 4 or 5, $t = 2{\cdot}23$, $2{\cdot}13$ or $2{\cdot}09$, respectively.

$C = 5d/6L_1$, $M = \frac{1}{2}d + (C.L_p)$ and $R =$ antilog M. M_L and M_U, the fiducial limits of M, are given by $\frac{1}{2}d + C(L_p \pm Q)$, where $Q = ts\sqrt{10n(3 + L_p^2/L_1^2)}$.

THE (4, 3) ASSAY DESIGN—

With the four standard preparation sums of n log-responses as y_1 to y_4 and the three test preparation sums as y_5 to y_7, the detached coefficients are as shown in Table IV.

TABLE IV

DETACHED COEFFICIENTS FOR (4, 3) ASSAY

	y_1	y_2	y_3	y_4	y_5	y_6	y_7	Standard error
L_p	−3	−3	−3	−3	+4	+4	+4	$s\sqrt{80n}$
L_1	−3	−1	+1	+3	−2	0	+2	$s\sqrt{28n}$
L'_1	−3	−1	+1	+3	+5	0	−5	$s\sqrt{70n}$
L_2	+3	−3	−3	+3	+2	−4	+2	$s\sqrt{60n}$
L'_2	+1	−1	−1	+1	−1	+2	−1	$s\sqrt{10n}$
L_3	−1	+3	−3	+1	0	0	0	$s\sqrt{20n}$

These are to be tested as before, with t having $7(n-1)$ degrees of freedom. When $n = 3$, 4 or 5, $t = 2{\cdot}15$, $2{\cdot}08$ or $2{\cdot}05$, respectively.

$C = 7d/6L_1$ and $M = \frac{1}{2}d + C.L_p$ as in the last example. The fiducial limits of M are given by $\frac{1}{2}d + C(L_p \pm Q)$, where $Q = 2st\sqrt{7n(3 + L_p^2/L_1^2)}$. The potency-ratio R and its fiducial limits are given by the antilogarithms of M and its fiducial limits as before.

NUMERICAL EXAMPLE—

A numerical example of this design follows to illustrate how the various calculations are carried through. In this, there were four tubes at each level (so that $n = 4$). The lowest dose of standard preparation was 2×10^{-10} g (0·0002 μg) of pure cobalamin, and the lowest dose of test preparation was 0·15 g of the material to be tested. Every other dose of either preparation was double the next lower dose, so that $d = \log_{10}2 = 0{\cdot}301$. The standard error, s, was estimated from the mean range as 15·0. The quantity t has $7 \times 3 = 21$ degrees of freedom and (for $P = 0{\cdot}05$) is 2·08. The sums of the four log-responses at each dose-level were—

(S.P.)—y_1	y_2	y_3	y_4	(T.P.)—y_5	y_6	y_7
408	582	820	1016	550	775	966

First, the various L values are calculated. For example,

$$L_p = -3(408 + 582 + 820 + 1016) + 4(550 + 775 + 966) = +686.$$

Its standard error is $15\sqrt{320} = 268$. It is thus significant, but not extremely so (686 divided by 268 = 2·56 as compared with $t = 2{\cdot}08$). L_1, the estimate of mean slope, is +2894, with standard error of 159, and is thus highly significant as it should be. $L_1' = -18$ with standard error of 250; $L_2 = -2$ with standard error of 232; $L_2' = +56$ with standard error of 95; $L_3 = -106$ with standard error of 134. All these are without significance and the assay is satisfactory. The significance of L_p may therefore be ignored.

$C = 2{\cdot}1/(6 \times 2894) = 0{\cdot}0001209$ and $M = 0{\cdot}1505 + 686C = 0{\cdot}2334$. $R =$ antilog M = 1·712, *i.e.*, the lowest dose of the test preparation (0·15 g) contains 1·712 times as much cobalamin as the lowest dose of standard preparation (2×10^{-10} g). From this, the mean potency of the test material is readily calculated as $22{\cdot}8 \times 10^{-10}$ g of cobalamin per g.

If the fiducial limits of the potency are required, one first computes

$$Q = 2 \times 15 \times 2{\cdot}08\sqrt{28(3 + 686^2/2894^2)} = 577{\cdot}3.$$

Then M_L and M_U are given by $0{\cdot}1505 + 0{\cdot}0001209(686 \pm 577{\cdot}3) = 0{\cdot}1636$ and 0·3032. Their antilogarithms R_L and R_U are 1·458 and 2·010, respectively, whence by the same process as was applied to R the fiducial limits of the potency are found to be 19·4 to $26{\cdot}8 \times 10^{-10}$ g of cobalamin per g.

REFERENCES

1. Wood, E. C., *Analyst*, 1946, **71**, 1.
2. Wood, E. C., and Finney, D. J., *Quart. J. Pharm.*, 1946, **19**, 112.
3. Bliss, C. I., *Ann. Math. Statist.*, 1946, **17**, 232.
4. Analytical Methods Committee, "Report on the Microbiological Assay of Riboflavine and Nicotinic Acid," *Analyst*, 1946, **71**, 397.
5. Wood, E. C., *Ibid.*, 1947, **72**, 84.
6. Pritchard, H., *Ibid.*, 1953, **78**, 460.
7. Wood, E. C., *Chem. & Ind.*, 1947, 334.
8. Finney, D. J., "Statistical Method in Biological Assay," Charles Griffin & Co., Ltd., London, 1952.
9. Lord, E., *Biometrika*, 1947, **34**, 41.

CLARENCE HOUSE,
CLARENCE ROAD,
NORWICH

December 18th, 1952

DISCUSSION

DR. J. I. M. JONES said that, notwithstanding the author's remarks on the difference between designs of microbiological assays for research and routine purposes, he must emphasise that every laboratory engaging in such assays had to pass through a research phase before it could settle down to a routine phase. This was so even when a procedure was taken from published methods, since it was essential that each laboratory should establish the nature of the response curve in its own environment. He also emphasised the need for every assay to be self-contained, *viz.*, that it should contain all the elements necessary to establish its own validity. Application of past experience to increase the precision was possible in only a few instances. Levels of response to the same dose differed on different occasions and so did slopes of response lines. Further, it would often be found that the variances differed significantly on different occasions. He quoted slopes ranging from 25·2 to 46·6 in 18 assays of cobalamin by the plate method with *E. coli*.

DR. WOOD said that he was in complete agreement with Dr. Jones—the result of an assay, its fiducial limits and an objective criterion of its validity should all be calculable without appeal to external evidence. Slopes, levels of response and variances did differ from assay to assay in almost all kinds of bio-assay; Fig. 2 of the present paper showed well the variations between six successive assays in one laboratory.

MR. J. P. R. TOOTILL said that increased replication on the standard (proportional to the square root of the number of unknowns) when assaying many samples against a common standard, as pointed out elsewhere by the author, would make the precision of the assay much less dependent on the value of the potency. It was not so important to put the unknown at approximately the same potency as the standard; an appropriate choice of standard doses in the centre of the linear range, so that the unknown even if higher or lower than expected would still be on the linear range, could be used without serious loss in precision.

DR. WOOD said that Mr. Tootill's remarks were no doubt applicable to the kind of multiple assay, almost always of the cup plate type, in which a large number of samples were assayed simultaneously by a Latin Square arrangement of doses. But when not more than three or four test preparations were assayed together in a tube assay, he still thought it dangerous to recommend to analysts without much statistical knowledge that the standard preparation doses should be confined to a small part of the linear range, particularly if there was any risk that the assay might prove to be invalid for one or more of the test preparations; there was less possibility of pitfalls if the standard preparation and the test preparation doses covered approximately the same part of the curve.

The Selection of Methods for Routine Assays for Members of the Vitamin-B Complex

BY H. PRITCHARD

(*Presented at the meeting of the Society on Wednesday, November 5th*, 1952)

Because of the difficulty of selecting suitable methods for the microbiological assay of members of the vitamin-B complex, the experience of a routine analytical laboratory is recorded. The methods described were adopted for thiamine, riboflavin, nicotinic acid, pantothenic acid, cobalamin, inositol and choline.

The methods for preparing the test materials for assay are indicated and the stock chemicals required for the preparation of the media are tabulated. The precision and inter-laboratory variation likely to be encountered are discussed.

THE chief advantages of microbiological as opposed to chemical methods of estimating members of the vitamin-B complex are that (*i*) they are sensitive and capable of estimating, in certain cases, materials present as only a few parts per thousand million; (*ii*) the vitamin being estimated needs only slight purification; (*iii*) the micro-organisms used are extremely selective and, under the right conditions, can be made to respond only to the vitamin whose assay is required; and (*iv*) they are capable of differentiating between biologically active and inactive forms, which would be difficult to do chemically.

METHODS AVAILABLE—

In the last ten years many methods of assay have been published, and test organisms that have been used successfully are listed in Table I.

TABLE I

TEST ORGANISMS THAT HAVE BEEN USED FOR THE ESTIMATION OF MEMBERS OF THE VITAMIN-B COMPLEX

Vitamin	Test organisms
Thiamine	*Saccharomyces cerevisiae*; *Phycomyces blakesleeanus*; *Lactobacillus fermentum*
Riboflavin	*L. helveticus*; *Leuconostoc mesenteroides*
Nicotinic acid	*L. arabinosus*; *Proteus vulgaris*
Pantothenic acid	*L. helveticus*; *L. arabinosus*
Pyridoxin	*S. cerevisiae*; *Neurospora sitophila*; *S. carlsbergensis*
Cobalamin (vitamin B_{12})	*L. lactis*; *Escherichia coli*; *L. leichmannii*; *Euglena gracilis*
Inositol	*S. cerevisiae*; *S. carlsbergensis*; *N. crassa* (inositol-less mutant)
Choline	*N. crassa* (choline-less mutant)
Folic acid	*L. helveticus*
Biotin	*L. arabinosus*; *Bacillus radicicola*; *N. crassa*
p-Aminobenzoic acid	*Acetobacter suboxidans*; *L. arabinosus*

Faced with such a diversity of methods the analyst often finds it difficult to select the one most suitable in particular circumstances. With a view to offering some guidance in this choice, the methods finally adopted by a routine laboratory after several years' work on the subject are described.

Official methods have not as yet been accepted for many microbiological methods. For example, in the "Bibliography of Standard Methods of Analysis" issued by the Society of Public Analysts and Other Analytical Chemists, microbiological methods are given only for thiamine, riboflavin and nicotinic acid. The seventh edition of "Methods of Analysis of the Association of Official Agricultural Chemists" gives methods for thiamine (by gas production), riboflavin, nicotinic acid and folic acid. Probably the most widely used source of information on methods of microbiological assay in this country is the collection of methods published by Barton-Wright.[1] In this book are described the estimations of ten members of the vitamin-B complex by methods selected and in some instances modified by the author. These are summarised in Table II.

TABLE II

METHODS RECOMMENDED BY BARTON-WRIGHT FOR ROUTINE ASSAYS

Vitamin	Test organism	End-point	Assay range, μg
Thiamine	*L. fermentum* 36	Turbidimetric	0·01 to 0·05
Riboflavin	*L. helveticus*	Titrimetric	0·05 to 0·25
Nicotinic acid	*L. arabinosus* 17/5	Titrimetric	0·05 to 0·3
Pantothenic acid	*L. arabinosus*	Titrimetric	0·005 to 1
Biotin	*L. arabinosus*	Titrimetric	0·00005 to 0·0005
Folic acid	*Streptococcus faecalis*	Titrimetric	1 to 10·0
Pyridoxin	*N. sitophila*	Weight of hyphae	0·1 to 0·8
p-Aminobenzoic acid	*N. crassa* (mutant 1633)	Weight of hyphae and plate	0 to 0·025
Choline	*N. crassa* (mutant 34486)	Weight of hyphae and plate	0 to 30·0
Inositol	*N. crassa* (mutant 37401)	Weight of hyphae and plate	0 to 30·0

Similar methods for thiamine, riboflavin, nicotinic acid, pantothenic acid and pyridoxin were recommended by Kent-Jones and Amos.[2]

Methods selected for use in this laboratory

The choice of the microbiological method finally adopted was influenced by the following considerations—

(*a*) It had to be simple and quick and involve the minimum of personal attention.
(*b*) It must not be too costly in chemicals or laboratory apparatus.
(*c*) It had to give reproducible results in the laboratory.

The methods adopted are listed in Table III.

For routine work the demands on this laboratory were mainly for assays for eight members of the vitamin-B complex, namely, thiamine, riboflavin, nicotinic acid, pantothenic acid, pyridoxin, cobalamin, inositol and choline. In selecting standard methods, many types of assay were closely examined and subjected to the trial of continuous experience.

Table III

Methods selected for use in the author's laboratory

Vitamin	Method	Test organism	Type of test	End-point	Assay range, μg
Thiamine ..	Jones and Morris[3]	*Lactobacillus fermentum* 36	Plate	Measurement	0·25 to 4·00
Riboflavin ..	Snell and Strong[4]	*L. helveticus*	Tube	Titrimetric	0·05 to 0·25
Nicotinic acid ..	Snell and Wright[5]	*L. arabinosus* 17/5	Tube	Titrimetric	0·05 to 0·3
Pantothenic acid	Hoag, Sarett and Cheldelin[6]	*L. arabinosus* 17/5	Tube	Titrimetric	0·005 to 1·0
Pyridoxin ..	Stokes, Larsen, Woodward and Foster[7]	*Neurospora sitophila*	Tube	Weight of hyphae	0·01 to 0·8
Cobalamin ..	Skeggs, Nepple, Valentik, Huff and Wright[8]	*L. leichmannii*	Tube	Titrimetric	0·000025 to 0·0002
	Bessel, Harrison and Lees[9]	*Escherichia coli*	Plate	Measurement	0·02 to 0·20
Inositol	Jones[10]	*Saccharomyces carlsbergensis*	Plate	Measurement	50 to 400
Choline ..	Horowitz and Beadle[11]	*N. crassa* (choline-less mutant)	Tube	Weight of hyphae	0 to 30

The riboflavin, nicotinic acid and pantothenic acid methods are the same as those described by Barton-Wright; these methods are very well established. In the pyridoxin estimation *N. sitophila* is used, but the method of extracting the vitamin has been modified.

In the estimation of thiamine by the tube method, it was found that the growth response of *L. fermentum* was poor and that results were much better when a plate method was used.

The assay of cobalamin presents some difficulty in that care has to be taken over *L. leichmannii* 313 when this culture is used. It is very sensitive to cobalamin and has many advantages; the strain is stocked by the National Collection of Industrial Bacteria (N.C.I.B. 8118). In this laboratory *L. leichmannii* is used for those materials containing low concentrations of the vitamin, such as feeding stuffs. For fish solubles and similar products, the *E. coli* plate method is excellent in that the culture appears to be very stable and the method can give a result quickly. The estimation of biotin has not yet been put on a sound footing, considerable difficulty having been met in removing traces of this vitamin from the medium; the organism recommended by Barton-Wright was very sensitive.

Preparation of the sample for assay—

It has been established that the method of preparing the sample for assay has considerable influence on the response to the test. The methods in use in this laboratory are, in most instances, those published by the various authors in describing the techniques adopted. With riboflavin, however, the results have been found here to be slightly higher when the samples have been steamed for half an hour. Pyridoxin is extracted by the method of

Morris, Herwig and Jones.[12] Cobalamin is extracted by the manner found least destructive, namely, by steaming the sample with an acetate buffer of pH 5·0 for half an hour, cooling, adjusting the pH to 6·8 and diluting and centrifuging in the usual manner.

CHEMICALS REQUIRED—

A laboratory that is prepared to carry out the microbiological assay of members of the vitamin-B complex must carry a stock of chemicals for making up the various media. The media are mostly prepared by mixing small quantities of highly purified amino-acids, purines, sugars and other compounds in various proportions to give a basal medium that is only lacking in the vitamin under test. The needs for the media are covered by some 43 chemicals, which are listed in Table IV together with sources of supply.

TABLE IV

CHEMICALS REQUIRED FOR THE MICROBIOLOGICAL ASSAY OF MEMBERS OF THE VITAMIN-B COMPLEX

Inorganic compounds
- Sodium acetate, hydrated
- Sodium citrate
- Sodium di-hydrogen citrate
- Sodium chloride, A.R.
- Potassium citrate (pure crystalline)
- Ammonium nitrate
- Ammonium sulphate
- Ammonium tartrate
- Salts A (phosphate buffer)
- Salts B (trace elements)

Organic compounds
- Citric acid
- Guanylic acid, sodium salt
- Thiomalic acid
- Tween 80

Sugars
- Glucose, A.R.
- Sucrose, A.R.
- Xylose-D (+)

Ureides
- Adenine
- Guanine
- Uracil
- Xanthine

Vitamins
- Thiamine
- Riboflavin
- Nicotinic acid
- Calcium-D-pantothenate
- Pyridoxin hydrochloride
- *p*-Aminobenzoic acid
- Biotin
- Folic acid

Amino-acids
- L-Cystine
- DL-Tryptophan

Supplements
- Hydrolysed casein "vitamin-free"
- Dehydrated Bacto-yeast extract
- Difco Standard Bacto-peptone

The inorganic compounds and citric acid, glucose and sucrose are obtainable from J. W. Towers & Co., Ltd.

Guanylic acid and thiomalic acid are obtainable from L. Light & Co., Ltd.

Tween 80 is obtainable from Honeywill & Stein Ltd.

The ureides and xylose-D (+), thiamine, nicotinic acid, *p*-aminobenzoic acid and tryptophan are obtainable from The British Drug Houses Ltd.

Riboflavin, calcium-D-pantothenate, pyridoxin hydrochloride, biotin, folic acid and L-cystine are obtainable from Roche Products Ltd.

Hydrolysed casein "vitamin-free" is obtainable from Allen & Hanbury Ltd.

Dehydrated Bacto-yeast extract and Difco Standard Bacto-peptone are obtainable from Baird & Tatlock Ltd.

Probably the most costly item of these is the guanylic acid, which is required for the medium used in estimating vitamin B_{12} by the tube method with *L. leichmannii*. Where any of the vitamins are to be used for standards, great care must be taken to see that they are pure and have indeed the potencies assumed. Riboflavin, thiamine and nicotinic acid standards can be obtained from the Medical Research Council. Finally, the water used must be glass-distilled and free from heavy metals.

PRECISION OF THE ASSAYS

A perfectly accurate method of assay would be independent of the type of organism used, and it must be accepted in the present state of knowledge that this does not hold for all these assays. Recently, for example, the inositol content of fish meal was estimated

with two different organisms, *S. carlsbergensis* and *N. crassa* (choline-less mutant); the former gave a value that was little more than a third of the latter. It cannot be stressed too highly, therefore, that not only must the method be stated when reporting a result, but also the organism used.

The selection of a very sensitive organism is of especial importance in the assay of feeding stuffs that contain antibiotic growth stimulants. These additives may influence the growth curve of the organism used (see Fig. 1). We have been able to estimate the amount of cobalamin (vitamin B_{12}) in a meal containing aureomycin by Skegg's method with an assay range of 0·000,025 to 0·0002 μg per tube. For riboflavin, the sensitive *Leuconostoc mesenteroides* 10,100 is to be commended; the assay range is 0·000,05 to 0·001 μg per tube.

As regards precision, a microbiological assay, like any bio-assay, demands as detailed and careful attention to manipulation and the standardisation of test conditions as is customary for chemical or physical assays. The method of extraction and of dilution, the conditions of incubation and the criterion of the end-point must all be assumed to affect the result; they must all, therefore, be controlled and standardised. Under optimum con-

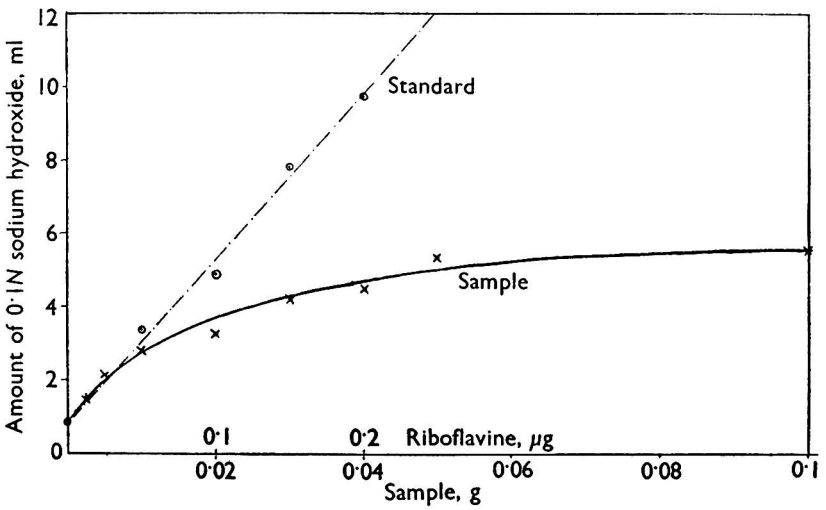


Fig. 1. Influence of antibiotic stimulants on growth

ditions of test in any one laboratory the response to the standard should remain fairly constant from assay to assay, but it must be borne in mind that such constancy does not guarantee the reliability of the assay and that an unusually high or low response to the standard does not necessarily mean that the assay is wrong.

A quantitative assessment of the precision to be expected can be made from results returned by different laboratories on duplicate samples of the same material. The agreement is usually satisfactory, as shown by the following typical results—

Vitamin	Laboratory A, μg per g	Laboratory B, μg per g
Riboflavin	5·9	4·1
Nicotinic acid	43·7	44·0
Pyridoxin	2·69	2·80
Vitamin B_{12}	0·117	0·120

The riboflavin results are the least reproducible, but as the values are very low, high precision cannot be expected.

The microbiological assay of riboflavin is probably the best and oldest established assay of the vitamins. The method introduced by Snell and his co-workers in 1939 is still valid, and the organism he used, *L. helveticus*, has proved stable and uniform. A series of assays of the riboflavin content of whale-liver meals carried out by Snell's method in three independent laboratories gave the results shown in Table V.

TABLE V

COMPARISON OF ASSAYS OF RIBOFLAVIN IN WHALE-LIVER MEALS BY SNELL'S METHOD

Sample	Laboratory A, μg per g	Laboratory B, μg per g	Laboratory C, μg per g
1	70·3	63·5	64·5
2	67·0	61·0	62·5
3	84·0	76·0	—
4	75·6	75·0	—
5	70·6	74·0	—
6	35·1	27·8	51·0
7	78·0	65·6	64·5

Examination of the various methods used in these laboratories showed that, although the same method of assay was used, laboratory A prepared the sample for assay by steaming the well-ground sample with acid for half an hour, whereas the other laboratories heated the sample in an autoclave for quarter of an hour. In view of this it is interesting and comforting to find from statistical analysis of the results that there is no evidence of a systematic difference between any of the laboratories. The same analysis reveals that the coefficient of variation of one result is about 14, *i.e.*, that in any long series of replications of an assay it can be expected—at least in these three particular laboratories—that about two-thirds of the results will fall within the range ±14 per cent. of the over-all mean.

REFERENCES

1. Barton-Wright, E. C., "The Microbiological Assay of the Vitamin-B Complex and Amino-Acids," Sir Isaac Pitman and Sons Ltd., London, 1952.
2. Kent-Jones, D. W., and Amos, A. J., "Modern Cereal Chemistry," The Northern Publishing Co., Ltd., Liverpool, 1947.
3. Jones, A., and Morris, S., *Analyst*, 1949, **74**, 333.
4. Snell, E. E., and Strong, F. M., *Ind. Eng. Chem., Anal. Ed.*, 1939, **11**, 346.
5. Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.
6. Hoag, E. H., Sarett, H. P., and Cheldelin, V. H., *Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 602.
7. Stokes, J. L., Larsen, A., Woodward, C. R., and Foster, J. W., *J. Biol. Chem.*, 1943, **150**, 17.
8. Skeggs, H. R., Nepple, H. M., Valentik, K. A., Huff, J. W., and Wright, L. D., *Ibid.*, 1950, **184**, 211.
9. Bessell, C. J., Harrison, E., and Lees, K. A., *Chem. Ind.*, 1950, 561.
10. Jones, A., *Analyst*, 1951, **76**, 588.
11. Horowitz, N. H., and Beadle, G. W., *J. Biol. Chem.*, 1943, **150**, 325.
12. Morris, S., Herwig, G., and Jones, A., *Analyst*, 1949, **74**, 37.

13, HAMILTON SQUARE
BIRKENHEAD, CHESHIRE

February 13*th*, 1953

DISCUSSION

DR. A. J. AMOS, in opening the discussion, complimented Mr. Pritchard upon an eminently practical paper, which presented information likely to be of considerable value to those new to the subject of microbiological assays. He was glad to find that, quite independently, he had arrived at the same selection of methods as had the author, except for cobalamin (vitamin B_{12}) assays, for which he was using the method of Emery, Lees and Tootill, whereas Mr. Pritchard had discarded this procedure in favour of the method of Skeggs *et al.* He asked what advantages Mr. Pritchard had found in this second method, apart from its use at lower concentrations.

He said that unlike the author, he had not met difficulties in freeing the biotin assay medium from biotin. He submitted his casein hydrolysate to two treatments with active charcoal and by so doing was able to attain blanks of the order of 1 ml of 0·1 *N* sodium hydroxide. With reference to stock cultures of *L. leichmannii*, Dr. Amos kept these on the M.T.Y. medium, sub-culturing daily, but also kept a reserve culture on a 10 per cent. suspension of freshly ground soya beans from which a sub-culture was prepared only once a month. If the daily sub-culturing of the M.T.Y. culture had to be discontinued for several days, as sometimes happened, it was possible to make a new M.T.Y. culture from the soya culture without loss of activity.

He agreed with Mr. Pritchard about the within-laboratory precision of an assay method that was being used frequently, and said that in his own laboratory he often attained fiducial limits of ±5 per cent. on assays of riboflavin and nicotinic acid. He could also confirm that agreement between laboratories was good in microbiological assays; he had that day turned up the results of some collaborative cobalamin

assays that he and Mr. Pritchard had made about two years previously. These figures revealed remarkably good agreement for both the tube and the plate methods, but at the same time they emphasised a point that had been made by other speakers, namely, that when the result of a microbiological assay was reported it was most important that the method used should also be reported. The figures showed that the *E. coli* plate method gave a significantly lower result than did the *L. leichmannii* tube method for cobalamin, although the agreement between laboratories for each of the assays was good.

MR. PRITCHARD said, in reply, that he found that the Skeggs method had several advantages over those published earlier, as well as greater sensitivity. It was possible, for example, to use titrimetric methods for the determination of the end-point. This was an advantage, as many laboratories had no facilities for accurate turbidimetric measurements. Also, the inoculum was more easily prepared. He also expressed his thanks for the description of Dr. Amos's method of removing biotin from the assay medium before the test.

DR. LESLIE HARRIS asked for information on one point. The author had said that the microbiological methods he had described were chosen because they "suited him well" and were reproducible. Dr. Harris asked what information was available, not about the reproducibility of the results, but about their "correctness." He asked for data, therefore, either from the work of Mr. Pritchard himself or from that of others, about the reliability of the methods as demonstrated by (*i*) agreement with other methods, including chemical and biological, (*ii*) freedom from interference by related or antagonistic influences and (*iii*) recovery of added vitamin.

MR. PRITCHARD replied that the methods adopted were, in the main, well established, and they had been well tried before they were published. Their reliability, therefore, was largely accepted. As for comparing these methods with chemical and biological ones, in many instances such methods were less sensitive and less accurate than microbiological techniques. Admittedly the microbiological results could be influenced by the presence of antagonistic substances such as antibiotics and also by the incomplete release of the vitamin from the material under test, but these influences could be overcome by the use of increasingly sensitive organisms, as described in the paper. Finally, the methods had been checked by recovery experiments before being adopted.

DR. G. E. FOSTER expressed surprise that the author did not give limits of error when reporting results of microbiological assays and asked if he could give some explanation of this omission.

MR. PRITCHARD said that limits of error were determined during the assay, but as a rule they were not reported because commercially a definite figure was needed and the statement of limits of error tended to confuse those who did not comprehend their significance. The most reliable figure possible was quoted.

MR. A. L. BACHARACH pointed out that the "short time" incubation techniques were only applicable to turbidimetric tube or plate exhibition methods or any others giving a direct estimate of bacterial multiplication. When, as in acidimetric procedures, a bacterial metabolite was being determined, it was essential to wait until the organisms had reached a plateau of production, as had been most elegantly demonstrated by McIlwain as long ago as 1947 (*Biochem. J.*, 1947, **41**, iii).

MR. PRITCHARD agreed that for titrimetric procedures the time of incubation was nearly always 72 hours.

DR. J. I. M. JONES emphasised that in the interpretation of the results of microbiological or biological assays one had to deal with much greater errors of random sampling than in chemical techniques, and that chemists were faced with a new discipline with which they must inculcate themselves. This involved statements of the probability of error of the result obtained. The mere statement of differences between the results found in two laboratories for the same sample was meaningless unless it was accompanied by a statement about the probable significance of the difference.

MR. C. H. ROBINS said that, as there was a certain narrow range of concentration outside which an assay would give no results, he wondered how much time was wasted in practice in attaining the correct dilution.

MR. PRITCHARD replied that in practice there were sufficient published figures to give guidance for a good nominal value to be obtained for particular substances. Where, however, mixtures or substances that had been treated in various ways were being examined, a wide range of dose levels should be tried to bracket the approximate potency and the result used as a nominal value for the accurate assay.

MR. J. P. R. TOOTILL said that limits of error were misleading unless the assay was known to be more accurate than it was precise.

MR. K. A. LEES pointed out that tube assay of vitamins gave a measure of growth-promoting ability, but that plate assays depended mainly on diffusion constants. A substance that was capable of giving only 70 per cent. of the maximal tube response might give 100 per cent. (or more or less) on plates, and the two methods did not necessarily correlate.

MR. PRITCHARD agreed with his view and pointed out that it had been well illustrated by the results mentioned by Dr. Amos.

Some Observations on the Determination of Benzoyl Peroxide in Flour and Bread

By R. A. KNIGHT and D. W. KENT-JONES

A comparison of the various methods and modifications used in the determination of benzoyl peroxide in flour and bread shows that low recoveries are given by the method of distillation and conversion to salicylic acid proposed by Nicholls in 1933.

In the distillation method the Mohler reaction is preferable to conversion into salicylic acid.

The highest recoveries are shown by extraction with ethyl ether, as described in the "Official Methods of Analysis," 7th edition, 1950, of the Association of Official Agricultural Chemists, followed by purification and absorptiometric determination by Mohler's reaction. This method is equally suitable for determining the benzoic acid in bread made from treated flour.

By the method proposed, the recovery from flour and bread is from 80 to 90 per cent.

Experiments showed that loss of benzoic acid during the baking of bread is insignificant and not more than 5 per cent. of the total.

Flour has been bleached with benzoyl peroxide for many years—at least thirty in this country—but only traces are used (about 15 p.p.m.). Because the amount of the substance in the flour is small, the analyst has encountered considerable difficulty in its determination. The greater part of the benzoyl peroxide added to flour decomposes into benzoic acid in a few days, but small amounts of peroxide persist for several weeks. Any method proposed for determining benzoyl peroxide in flour must, therefore, include unchanged peroxide as well as the benzoic acid derived from it.*

A method for the determination of benzoyl peroxide in flour on which cereal chemists in this country have in the past relied was proposed by Nicholls in 1933.[1] Briefly, the treated flour is steam-distilled in the presence of calcium chloride, the benzoic acid extracted from the distillate, any free benzoyl peroxide converted into benzoic acid by treatment with acetone and sodium hydroxide solution and the benzoic acid, after treatment with permanganate, is converted into salicylic acid, the colour reaction of which with ferric chloride is then relied upon. As was shown by Nicholls,[2] only about 10 per cent. of the benzoic acid is converted, under the conditions of the test, into salicylic acid, so that in dealing with 1 mg of benzoic acid the determination has to be carried out on approximately 0·1 mg of salicylic acid.

It is not surprising, therefore, that the determination of benzoyl peroxide in flour has been subject to appreciable experimental error. The commercial flour-bleacher in use contains 15 per cent. of pure benzoyl peroxide and is often used in quantities as low as half an ounce to a sack of 280 lb of flour, *i.e.*, there is only about 1 part of benzoyl peroxide in 60,000 parts of flour. A series of benzoyl peroxide determinations was made in 1939 by four experienced analysts in different laboratories; all used the Nicholls technique. Their results were, in general, disturbing, in that they were low and erratic.

Discrepancies between different laboratories shown by the distillation - salicylic acid method may be due to incomplete distillation of the benzoic acid, to the fact that there is only partial conversion of the benzoic acid to salicylic acid or to the uncertainty of the subsequent colour reaction. It may also be that the success of the recovery is partly dependent on the precise conditions of distillation, size of sample, flask, etc.

As a finish to the distillation method, we prefer Mohler's method, as modified by Illing,[3]

* For the detection of unchanged benzoyl peroxide in flour that does not contain either bromate or persulphate, the following test was found satisfactory. Make 20 g of flour into a thin paste with 10 per cent. potassium iodide solution in a boiling-tube. Immerse the tube in boiling water for 5 minutes, stirring occasionally, and spread the paste thinly on a glass plate. Examine the paste with a hand lens. Benzoyl peroxide is indicated by brownish-blue specks, about ½ to 1 mm in diameter. These may be confirmed by viewing them through a low-power microscope.

to the conversion to salicylic acid. This method relies on the red colour of *m*-diaminobenzoic acid, produced by nitration of benzoic acid to the *m*-dinitro derivative and reduction with hydroxylamine in ammoniacal solution. Although, in our hands, the recoveries of benzoyl peroxide from synthetic samples have been no better by one method than by the other, we have found that the colour given by Mohler's reaction is more easily matched against the standards than is that of ferric salicylate.

In the 7th edition of the A.O.A.C. "Methods of Analysis" a new procedure is recommended for the determination of benzoyl peroxide in flour.[4] In essence, this consists in extracting the flour (50 g) with ethyl ether, reducing residual benzoyl peroxide with iron and hydrochloric acid, purifying and determining the benzoic acid by the Mohler reaction. The absorption of the final solution is measured within 30 minutes by a photo-electric absorptiometer through green filters having a maximum transmission at 510 mμ. A standard series of benzoic acid solutions is treated in the same way as the flour extracts and a standard graph is prepared. It is necessary to prepare a standard graph for each set of determinations and make allowance for any blank, *i.e.*, for any colour given by a known untreated flour. The use of the recommended Nuchar W (an activated carbon) to effect additional purification was found to be unnecessary for ordinary bakers' flours. We have made numerous tests by this procedure and for the first time we have been able to get high recoveries, of the order of 90 per cent., when the benzoyl peroxide was present to the extent of about 16 p.p.m.

Since, in bread made with flour containing benzoyl peroxide of this order, we have never been able to recover more than 45 per cent. by the distillation - salicylic acid method, even with the more sensitive Mohler reaction in the final stages of the determination, the new procedure seems to be particularly suitable in the examination of bread.

In our tests we used the commercial 15 per cent. benzoyl peroxide product to the extent of half an ounce per sack, *i.e.*, 16·7 p.p.m., in the flour and baked bread from this treated flour. The powdered crumb was air-dried and ground to pass a 5 XX silk screen (0·064 sq. mm mesh). Fifty grams of the fine powder were treated by the A.O.A.C. method for flour, except that the second extraction with ether was prolonged overnight.

A typical result is shown in Table I.

TABLE I

RECOVERY OF BENZOYL PEROXIDE, AS BENZOIC ACID, FROM BREAD MADE FROM BLEACHED FLOUR, BY THE A.O.A.C. METHOD

Benzoyl peroxide (as benzoic acid) added to original flour (13 per cent. moisture)	=	16·9 p.p.m.
Benzoic acid found in treated loaf (calculated to 13 per cent. moisture)	=	16·7 p.p.m.
Apparent benzoic acid in untreated loaf (calculated to 13 per cent. moisture)	=	2·4 p.p.m.
Recovery of added benzoyl peroxide (allowing for salt and yeast in bread)	=	$\frac{(16{\cdot}7 - 2{\cdot}4)}{16{\cdot}9} \times 100 \times \frac{670}{655} = 87\%$

(there were 15 g of salt and yeast solids added to the 655 g of flour).

NOTE—There is always a slight absorption of light in the extract from untreated flour. The solution is faintly yellow, but has not the characteristic red colour given by traces of benzoic acid.

This result confirms that there is little loss of benzoic acid during baking, as the recovery on the flour used to make this bread was 93 per cent.

Typical results by different methods for the determination of benzoyl peroxide in treated flour and bread are shown in Table II.

As the recoveries attained were only in the region of 90 per cent. it became necessary to find out whether the failure to recover the full amount could be ascribed to loss of benzoic acid by steam distillation during the baking process. To test this point, we added to a flour larger quantities of benzoic acid than the residual amounts normally resulting from the benzoyl peroxide bleaching of flour on a commercial scale. A flour was treated with benzoic acid in a proportion equivalent to the use of 4 oz of commercial (15 per cent. benzoyl peroxide) bleacher per sack and 100 g of the flour made into a yeasted dough, fermented for 1 hour, "knocked back" and then introduced into a litre flask fitted with a condenser and receiver. After a further hour the dough was baked at 450° F for 1 hour by immersing the flask in a heated

oil-bath and the distillate was collected in an excess of sodium hydroxide. The alkaline solution was evaporated to a small bulk, treated with permanganate and the excess of the latter was removed with sulphur dioxide. After acidification, the liquid was extracted three times with ether and the combined ether extracts were evaporated cautiously to dryness at 30° C. Any benzoic acid in the residue was determined by the Mohler reaction.

In three such experiments quantities of benzoic acid were recovered that varied between 2 and 5 per cent. of the amount present in the original flour. The loss of benzoic acid in baking is therefore likely to be small and certainly not more than 5 per cent. of the amount originally present. As it is sometimes suggested that there is appreciable loss of benzoic acid in baking, it is useful to record this experiment.

TABLE II

RECOVERY OF BENZOIC ACID FROM FLOUR AND BREAD

All recoveries on treated flour are corrected for the apparent benzoic acid content of the untreated flours

Weight of sample taken g	Flour treatment	Method	Recovery, %	
	FLOUR			
250	1 oz/sack 15 per cent. benzoyl peroxide	Nicholls	30	A larger flask than usual was used for working with the larger sample
250	½ " "	"	24	
250	½ " "	Nicholls (Mohler)	43	
100	1 " "	Nicholls	45	
50	½ " "	Nicholls (Mohler)	77	
50	10 p.p.m. benzoic acid	Nicholls	60	Blank on untreated flour, 4 p.p.m.
50	20 "	"	55	
50	10 "	Nicholls (Mohler)	54	Blank on untreated flour, 3·2 p.p.m.
50	20 "	"	61	
50	20 "	"	45	Blank on untreated flour, 2 p.p.m.
50	10 "	A.O.A.C.	82	Blank on untreated flour, 2 p.p.m.
50	20 "	"	90	
50	½ oz/sack 15 per cent. benzoyl peroxide	" *	93	Blank on untreated flour, 3·8 p.p.m.
50	½ " "	"	82	
	BREAD			
100	½ oz/sack 15 per cent. benzoyl peroxide	Nicholls (Mohler)	35	
100	1 " "	"	45	
50	½ " "	A.O.A.C.*	87	Blank 2·5 p.p.m.
50	½ " "	"	91	Blank 1·8 p.p.m.

* In both experiments marked with an asterisk the same flour was used.

CONCLUSIONS

It has been shown that the A.O.A.C. method for the determination of benzoyl peroxide in flour is satisfactory and that high recoveries are possible. Furthermore, it appears that the method is well suited to, and may be used for, bread made from flour treated by benzoyl peroxide. It has been demonstrated that the loss of benzoic acid on baking is slight.

REFERENCES

1. Nicholls, J. R., *Analyst*, 1933, **58**, 4.
2. ——, *Ibid.*, 1928, **53**, 19.
3. Illing, E. T., *Ibid.*, 1932, **57**, 224.
4. The Association of Official Agricultural Chemists, "Official Methods of Analysis," Seventh Edition, Washington, U.S.A., 1950, p. 205.

THE LABORATORIES
DUDDEN HILL LANE
WILLESDEN, N.W.10

February 13th, 1953

A Rapid Method for the Determination of Molybdenum in Soils

By J. L. GRIGG

A rapid method for determining total and available molybdenum in soils has been developed. Interference by chromium, titanium and vanadium in the molybdo-thiocyanate method has been prevented. Chromium is removed as chromyl chloride by evaporation with hydrochloric acid in the presence of perchloric acid. A complex of titanium that does not colour the amyl alcohol is formed with sodium fluoride. The amyl alcohol layer is washed twice with stannous chloride solution, the first time to remove the iron and the second to reduce vanadium to a form in which its interference is negligible. Results compare favourably with the ether separation method.

The method used in these laboratories for separating molybdenum from those elements in soil extracts that interfere in its determination as molybdo-thiocyanate[1] has been to extract the molybdenum together with ferric iron from solution in constant boiling-point hydrochloric acid with diethyl ether. As the partition coefficient of molybdenum between ether and this strength of acid is only about 0·7, four extractions with ether are necessary for complete separation of the molybdenum. The combined ether fractions are then washed 3 or 4 times with water to return the molybdenum to the aqueous phase for absorptiometric determination.

This procedure is time-consuming when done on a routine scale. To enable large numbers of samples to be analysed, a more rapid method has been sought that would avoid the ether separation.

The only other reagent for molybdenum of sufficient sensitivity is toluene-3:4-dithiol. This has been used successfully for the determination of molybdenum in plant material[2] and in steel.[3] Wells and Pemberton[3] attained excellent results with this reagent for steels containing 0·01 to 4·22 per cent. of molybdenum in a 4-mg sample. When their method was applied to soil samples, the amount of iron present, 100 to 500 mg, interfered with the determination, as it was partly extracted by the amyl acetate. The method was therefore not studied further.

Those elements that are likely to be present in soil extracts and that would interfere in the molybdenum determination by Perrin's method[1] if the ether separation were omitted are titanium, vanadium and chromium. When the molybdo-thiocyanate is extracted into amyl alcohol to concentrate and stabilise the colour for photometric determination, it is found that titanium and vanadium give interfering yellow colours and chromium a blue colour.

From published analytical figures the amounts of these elements likely to occur in soil extracts have been calculated and are shown in Table I, together with the amount found by Perrin[4] to give interference equivalent to 0·3 μg of molybdenum. The ignited residue of an oxalate extract of 25 g of soil as prepared for the determination of available molybdenum weighs from 1 to 1·5 g. If a proportional extraction of titanium, vanadium and chromium is assumed, the maximum amounts to be expected are 13·5 mg of titanium, 1·5 mg of vanadium and 7·5 mg of chromium, all well above the permitted tolerance level.

TABLE I

INTERFERING ELEMENTS IN THE DETERMINATION OF MOLYBDENUM BY THIOCYANATE

Element	Possible proportion in soil, p.p.m.	Reference	Amount in 5-g sample of soil, mg	Amount to cause error equivalent to 0·3 μg of molybdenum,* mg
Titanium	>1000	Mitchell[5]	>5	0·7
	up to 9000	Askew[6]	45	
Vanadium	20 to 1000	Mitchell[5]	0·1 to 5	0·3
Chromium	10 to 5000	Mitchell[5]	0·05 to 25	0·7

* According to Perrin.[4]

PREVENTION OF INTERFERENCE—

Methods of obviating the interference of these ions were studied. A likely method would be the formation of complex ions that were colourless in amyl alcohol. Other possibilities were removal of the interfering elements by selective extraction of complexes, or removal by volatilisation.

Titanium—Dick and Bingley[7] found that if sodium fluoride was added to the solution before development of the molybdo-thiocyanate colour, interference from titanium was reduced. This method was successfully tried on standard titanium solutions. The interference rises to a maximum with about 10 mg of titanium. As described by Dick and Bingley, 2 ml of 5 per cent. sodium fluoride reduced the interference considerably. Trials with different amounts of sodium fluoride indicated that 5 ml of a 5 per cent. solution completely decolorised 30 mg of titanium, whilst 10 ml introduced a slight turbidity (Table II). Consequently, 5 ml was accepted as the optimum amount to remove titanium interference.

TABLE II

SUPPRESSION OF TITANIUM INTERFERENCE BY SODIUM FLUORIDE

Titanium present, mg	Sodium fluoride, 5 per cent., added, ml	No molybdenum present		10 μg of molybdenum present	
		Optical density	Molybdenum equivalent, μg	Optical density	Molybdenum equivalent, μg
0	0	0·010	0·0	0·162	10·0
30	0	0·093	4·8	0·251	14·0
30	2	0·019	0·4	0·171	10·5
30	5	0·010	0·0	0·165	10·2
30	10	0·022*	0·6	0·185*	11·4

* Turbid solution.

Vanadium—Attempts to complex vanadium with citric acid failed to prevent interference. In work with standard solutions it was found that, in the absence of iron, vanadium did not interfere, although in the presence of iron the yellow colour was formed. A study of standard oxidation - reduction potentials offers an explanation of this. From Table III it is seen that the stannous chloride used to reduce sexavalent molybdenum to the quinquivalent state is powerful enough to reduce vanadate to tervalent vanadium, which is not extracted into amyl alcohol. When iron is present in fairly large amounts, however, the reduction does not proceed completely to the tervalent state, some vanadyl chloride being formed instead, and this appears to be the compound producing the interference.

TABLE III

STANDARD OXIDATION - REDUCTION POTENTIALS

		Volts
$Sn^{\cdots\cdot}$	$\rightarrow Sn^{\cdot\cdot}$	−0·13
$Fe^{\cdots}$	$\rightarrow Fe^{\cdot\cdot}$	−0·77
$(Mo^{VI}O_4)''$	$\rightarrow Mo^{V}$	−0·4
$(V^{V}O_3)'$	$\rightarrow (V^{IV}O)^{\cdot\cdot}$	−1·00
$(V^{IV}O)$	$\rightarrow V^{\cdots}$	−0·31

On this theory it was found that if the amyl alcohol extract was washed with dilute stannous chloride once to remove iron and a second time to reduce vanadyl chloride to vanadic, then no interference occurred (Table IV). A double wash with a 1 in 25 dilution of the 40 per cent. stannous chloride was therefore included in the method to prevent vanadium interference.

Chromium—Chromium interference was not prevented by the fluoride or double stannous chloride wash. Smith[8] removed chromium from stainless steel samples by volatilisation as chromyl chloride, b.p. 167° to 168° C. His method was to evaporate the 72 per cent. perchloric acid solution several times with hydrochloric acid, chromyl chloride being evolved as a reddish vapour.

This technique was tried with good results. An amount of potassium dichromate equivalent to 5 mg of chromium was treated with 1 ml of 72 per cent. perchloric acid and

5 ml of hydrochloric acid, evaporated to dryness and the evaporation repeated. Complete volatilisation of chromium was attained in presence or absence of iron (Table V). This evaporation was incorporated in the method.

TABLE IV

PREVENTION OF INTERFERENCE BY VANADIUM

5 mg of vanadium present

Method	No iron present		170 mg of iron present	
	Optical density	Molybdenum equivalent, μg	Optical density	Molybdenum equivalent, μg
No stannous chloride wash ..	0·041	1·7	0·102	5·3
1 stannous chloride wash ..	0·014	0·1	0·053	2·5
2 stannous chloride washes ..	0·011	0·0	0·012	0·0

TABLE V

REMOVAL OF CHROMIUM BY VOLATILISATION

5 mg of chromium as potassium dichromate treated as shown

Treatment	Optical density	Molybdenum equivalent, μg
None	0·075	3·7
Evaporated twice with 5 ml of HCl	0·019	0·4
Evaporated twice with 5 ml of HCl + 1 ml of $HClO_4$	0·016	0·2
Evaporated twice with 5 ml of HCl + 1 ml of $HClO_4$ (200 mg of iron present)	0·013	0·0

METHOD

Total molybdenum—Total molybdenum is determined on 5 g of soil that has been ashed at 450° C and treated with hydrofluoric acid to remove silica, the residue being dissolved in hydrochloric acid and the solution filtered and evaporated to dryness.

PROCEDURE—

Dissolve the residue in 5 ml of constant boiling-point hydrochloric acid and 1 ml of 72 per cent. perchloric acid. Evaporate this solution to dryness and repeat the evaporation.

Cool the residue and dissolve it in exactly 5 ml of constant boiling-point hydrochloric acid and transfer the solution with the aid of the minimum quantity of water to a glass-stoppered test tube graduated at 32 ml. Add 5 ml of 5 per cent. sodium fluoride solution, 1·5 ml of 20 per cent. potassium thiocyanate and 3 ml of 40 per cent. stannous chloride in hydrochloric acid (1 + 9) and then dilute to 32 ml. Add 10 ml of *iso*amyl alcohol, insert the stopper and shake the tube. Place it in a bath of water at 25° C for 20 minutes.

Remove the aqueous phase with a suction pipette and shake the amyl alcohol layer with 25 ml of a 1 in 25 dilution of the 40 per cent. stannous chloride reagent. Replace the tube in the bath until all the iron colour has faded. Remove the aqueous phase and shake the alcoholic layer with a second 25-ml portion of the diluted stannous chloride. Separate the aqueous layer as completely as possible and filter the amyl alcohol solution through a dry Whatman No. 1 filter-paper into a dry tube.

Measure the absorption of the solution in a 50-mm micro-cuvette with a spectrophotometer at a wavelength of 465 mμ.

Available molybdenum—Available molybdenum is extracted by shaking 25 g of air-dry soil with 250 ml of acid ammonium oxalate buffer (498 g of ammonium oxalate and 252·1 g of oxalic acid per 20 litres) at 25° C overnight.[9] The extract is filtered and a measured aliquot of the filtrate is evaporated to dryness in a silica beaker and ignited for 3 to 4 hours at 450° C to remove oxalate and organic matter. The ignited residue is then treated as described in the procedure.

Results

This method was found to give results closely comparable with those of Perrin's ether separation technique when a number of soils were tested for available molybdenum (Table VI).

Table VI

Comparison of ether separation and rapid methods for available molybdenum

Molybdenum, p.p.m. on air-dry soil

Ether method	Rapid method	Ether method	Rapid method	Ether method	Rapid method
0·34	0·31	0·10	0·12	0·13	0·08
0·10	0·10	0·12	0·11	0·08	0·09
0·33	0·35	0·31	0·31	0·09	0·07
0·06	0·07	0·22	0·21	0·03	0·06
0·21	0·19	0·15	0·13	0·05	0·04

Statistical analysis shows that the differences between the two groups are not significant.

The use of test tubes and a suction pipette rather than separating funnels permits the aqueous and amyl-alcohol phases to be separated at a constant temperature in a water-bath, so minimising differences due to the solubility of the amyl alcohol.

The recovery of added molybdenum was tested by adding 5 μg of molybdenum to four soil extracts. The amounts recovered were 5·1, 5·0, 5·2 and 5·0 μg, which indicated complete recovery and only very slight interference from other elements.

The time required for a complete determination once the extracts have been prepared is about 3 hours. The previous method required 8 to 9 hours for the ether separation, removal of dissolved ether and colorimetric analysis.

References

1. Perrin, D. D., *N.Z. J. Sci. Tech., Sect. A*, 1947, **28**, 183.
2. Piper, C. S., and Beckwith, R. S., *J. Soc. Chem. Ind.*, 1948, **67**, 374.
3. Wells, J. E., and Pemberton, R., *Analyst*, 1947, **72**, 185.
4. Perrin, D. D., *N.Z. J. Sci. Tech., Sect. A.*, 1946, **27**, 396.
5. Mitchell, R. L., Technical Communication No. 44, Commonwealth Bureau of Soil Science, **1948.**
6. Askew, H. O., *N.Z. J. Sci. Tech.*, 1930, **12**, 173.
7. Dick, A. T., and Bingley, J. B., *Aust. J. Exp. Biol. Med. Sci.*, 1951, **29**, 459.
8. Smith, T. W., *Ind. Eng. Chem., Anal. Ed.*, 1938, **10**, 360.
9. Grigg, J. L., *N.Z. J. Sci. Tech., Sect. A.*, 1953, **34**, 405.

Galloway Laboratories
Rukuhia Soil Research Station
Hamilton, New Zealand

January 23rd, 1953

The Determination of Keten and Acetic Anhydride in the Atmosphere

By W. M. DIGGLE and J. C. GAGE

Keten or acetic anhydride can be determined in air by absorption in alkaline hydroxylamine, treatment with acid ferric chloride and photoelectric measurement of the ferric acetohydroxamic acid complex.

Keten can be determined in the presence of acetic anhydride by passing the air first through an absorber containing toluene, which absorbs the anhydride.

Keten is a colourless gas with a characteristic odour resembling that of acetic anhydride; there is a wide variation in the ability of different individuals to detect this odour in the

atmosphere. It is a highly toxic gas; the symptoms produced by inhaling excessive amounts resemble those caused by phosgene[1,2,3] and it has been stated[1] that as little as 1 part per million produces definite toxic symptoms in experimental animals. Certain industrial processes require the use of considerable amounts of keten, so this investigation was undertaken to discover a method for determining it in the atmosphere.

Keten reacts with hydroxyl and amino groups to form acetyl derivatives. The only methods given in the literature for the determination of keten in air are based on its reaction with a large excess of aniline in aqueous solution and the subsequent gravimetric determination of the precipitated acetanilide[2] or on its reaction with an excess of sodium hydroxide to form sodium acetate and titration of the excess of sodium hydroxide.[3] These methods are

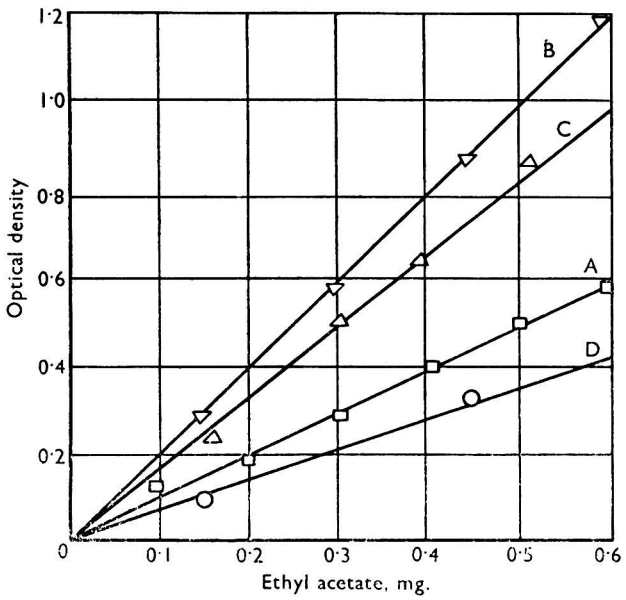


Fig. 1. Standard graphs for calculating keten and acetic anhydride concentrations. A, ethyl acetate; B, keten; C, keten (one absorber); D, acetic anhydride

useless for the determination of less than 1 part of keten per million of air, as they would require excessively large volumes of air. In our search for a method based on the change in colour or colour intensity of a dyestuff after reaction with keten, we have found that the colour or potential colour of a dyestuff or chromogenic substance is usually inhibited by acetylation. As we have shown that keten is not very soluble in organic solvents, and as efficient absorption of keten from the atmosphere is only achieved in liquids with which keten reacts, the colorimetric procedure in such a method must involve the measurement of a small colour change at a high colour intensity. An exception to this generalisation is the reaction of keten with hydroxylamine, which gives acetohydroxamic acid, the ferric complex of which has a purple colour. The optimal conditions for this general reaction for acetyl groups have been studied by Hestrin.[4]

Experimental

Reagents—

Hydroxylamine hydrochloride solution, 20 *per cent. w/v.*

Sodium hydroxide solution, 20 *per cent. w/v.*

Alkaline hydroxylamine reagent—Equal volumes of the 20 per cent. w/v hydroxylamine hydrochloride and sodium hydroxide solutions were mixed shortly before use. Hydroxylamine, particularly in alkaline solution, may produce dermatitis, and care should be taken to prevent it from coming into contact with the skin.

Acid ferric chloride reagent—A 10 per cent. w/v solution of hydrated ferric chloride was made in a mixture of equal parts by volume of concentrated hydrochloric acid, sp.gr. 1·18, and water.

PREPARATION OF TEST KETEN ATMOSPHERES—

Keten was prepared by the pyrolysis of acetone in the apparatus described by Williams and Hurd[5] and absorbed in a spiral bubbler containing dry acetone. The keten content of this solution, after dilution with dry acetone, was determined by adding a measured volume of it to absolute ethanol purified by distillation over potassium hydroxide; the ethyl acetate produced was determined by adding to 5 ml of the ethanol solution 4 ml of alkaline hydroxylamine and then, after 1 minute, 5 ml of acid ferric chloride reagent. The optical density of the resulting mixture, less the optical density of a reagent blank measured by subjecting 5 ml of the purified ethanol to the same procedure, was compared on a standard graph constructed by similarly treating a series of standard solutions of ethyl acetate in ethanol; this standard graph, A, is shown in Fig. 1. By this means the ethyl acetate content of the test solution, and from this the keten content of the acetone solution, was calculated. The output of the keten pyrolyser was found to be of the order of 50 mg per minute. As solutions of keten in acetone are not highly stable they were rejected after 2 hours.

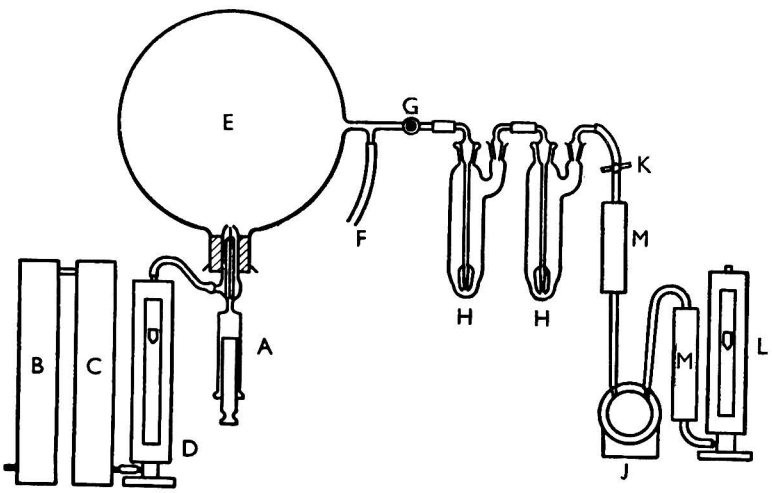


Fig. 2. Diagram of apparatus for preparing and sampling test keten and acetic anhydride atmospheres

The test keten atmospheres were prepared by vaporising the standardised keten solution, appropriately diluted with dry acetone, into a metered stream of air in the apparatus shown diagrammatically in Fig. 2. The keten solution was contained in the glass syringe forming part of the controlled fluid feed atomiser, A, previously described[6]; as some difficulty was experienced in maintaining a polythene to glass connection in the presence of acetone, the atomiser was sealed directly to the glass syringe. The syringe drive mechanism delivered the keten solution at a fixed rate of 0·0367 ml per minute into an air stream, which had been filtered and dried on passing through the calcium chloride and silica gel towers, B and C, respectively, and which was controlled at a fixed rate of 10 litres per minute as measured by the rotameter, D, which was calibrated at the working pressure. The solution of keten in acetone was diluted with dry acetone to produce atmospheres containing from 1 to 6 mg of keten per cubic metre. The atomiser delivered the atmosphere into the mixing chamber, E, of 2 litres capacity, from which it passed to the waste duct, F.

DETERMINATION OF KETEN IN THE TEST ATMOSPHERE—

No liquid has been discovered which, although not reactive with keten, is a sufficiently good solvent to remove it from the atmosphere for the purpose of air analysis. Nor do

liquids such as ethanol, which by virtue of its hydroxyl group is capable of reacting with keten, react sufficiently rapidly to be used for this purpose. We have found, however, that the alkaline hydroxylamine reagent is suitable; it retains a large proportion of the keten from the air sample passed through it.

In order to determine the extent to which keten reacts with the alkaline hydroxylamine, 0·1-ml portions of various dilutions of the standardised acetone solution of keten were added to 10 ml of the alkaline hydroxylamine. After 1 minute, 5 ml of acid ferric chloride reagent were added and after 30 minutes the optical density was measured at 540 mμ, by means of a Unicam DG spectrophotometer incorporating 1-inch cylindrical cells. The blank value for the reagents alone was found by submitting 0·1 ml of acetone to a similar procedure. The standard graph, B, is shown in Fig. 1. A standard graph, A, similarly obtained from 0·1-ml portions of a series of solutions of ethyl acetate in acetone, is also shown in Fig. 1; this is identical with the standard graph used when analysing the keten solutions. The ratio of the slopes of the ethyl acetate graph, A, and the keten graph, B, is 0·492; as the ratio of their molecular weights is 2·10, the percentage of keten that reacted was 96·8.

In order to determine the keten content of the test atmosphere, two absorbers of the type described by Gage,[7] calibrated at 15 ml, were connected in series to the apparatus at the sampling stopcock, G. Into each absorber was measured 5 ml of the hydroxylamine hydrochloride and sodium hydroxide solutions. The stopcock, G, was opened and air sampled through the absorbers for 15 minutes at a rate of 6 litres per minute. Air was drawn through the absorbers, H, by means of the vacuum pump, J, the rate of sampling being controlled by means of the screw-clip, K, and measured by the rotameter, L. The pump and rotameter were protected by the glass-wool filters, M. This method of controlling the sampling rate is convenient as the rotameter operates at atmospheric pressure and does not need special calibration, but its use depends on the pump being so designed that the volume of free air entering it under the experimental conditions is the same as the volume leaving it; these volumes must be checked with two flowmeters, one on either side of the pump. If the pump does not permit this type of sampling-rate control, the rotameter must be situated between the absorbers and the pump and, as there is a considerable pressure drop across the two absorbers, the rotameter must be calibrated to give the correct rate at the working pressure.

At the end of the sampling period the absorbers were detached, the tops raised and the gas entry tube washed with 5 ml of ferric chloride reagent. The volume was adjusted to 15 ml with distilled water and the optical density was measured as described above. The measured optical density, less the blank value for the reagents, was applied to the standard graph, B (Fig. 1), and the keten content of the sample was calculated. Table I shows the efficiency of sampling; the optical densities of the solutions in the first absorber have been plotted against the total keten in the air sample in Fig. 1 (C). From the ratio of the slopes of graphs C and B, the retention in the first absorber is 82·6 per cent.

TABLE I

EFFICIENCY OF SAMPLING TEST KETEN ATMOSPHERES

Concentration of keten, mg per cu. metre	Volume of air sampled, litres	Keten absorbed		Calculated concentration of keten, mg per cu. metre
		by first absorber, mg	by second absorber, mg	
1·09	90	0·092	0·012	1·15
1·82	90	0·163	0·015	1·96
3·19	90	0·247	0·053	3·34
4·42	90	0·338	0·063	4·46
5·66	90	0·437	0·098	5·95

INTERFERENCE BY ACETIC ANHYDRIDE—

Carboxylic acids do not interfere with the determination of keten by the above method, but the reaction is given by their acid chlorides, anhydrides and esters. Of these, the only substance likely to co-exist in air with keten is acetic anhydride, which may be used in industrial processes involving keten, or to which keten may be converted in moist air.

The reactivity of acetic anhydride with alkaline hydroxylamine was determined in a manner similar to that used for keten; the optical densities of the colours developed from a

series of solutions in acetone were measured. The standard graph, D, is shown in Fig. 1; the ratio of the slopes of the ethyl acetate graph, A, and the acetic anhydride graph, D, is 1·42, and as the ratio of the molecular weights is 0·863, the percentage of acetic anhydride that reacts is 81·5. A series of mixtures of acetic anhydride in air was prepared and analysed as described for keten; the measured optical densities were applied to graph D, Fig. 1, and the results are shown in Table II.

TABLE II

EFFICIENCY OF SAMPLING TEST ACETIC ANHYDRIDE ATMOSPHERES

Concentration of acetic anhydride in air, mg per cu. metre	Volume of air sampled, litres	Acetic anhydride absorbed		Calculated concentration of acetic anhydride, mg per cu. metre
		by first absorber, mg	by second absorber, mg	
2·14	90	0·200	0	2·22
3·21	90	0·283	0	3·14
4·28	90	0·413	0	4·60
7·23	90	0·660	0	7·35
10·3	90	0·985	0	11·0
15·0	90	1·37	0	15·2
21·7	90	1·87	0	20·8

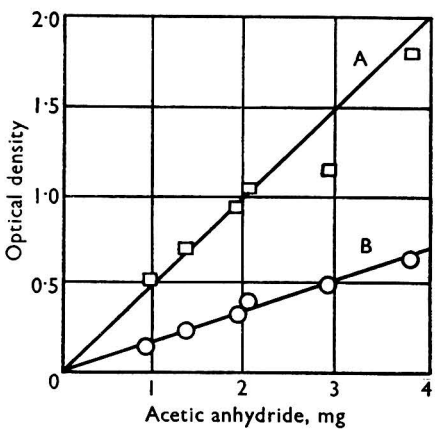


Fig. 3. Partition of acetic anhydride vapour between toluene and alkaline hydroxylamine. A, standard graph for toluene absorber; B, standard graph for hydroxylamine absorber

DETERMINATION OF KETEN IN THE PRESENCE OF ACETIC ANHYDRIDE—

When air containing acetic anhydride vapour is passed through toluene, a large part of the acetic anhydride is absorbed, but keten is not retained to a significant extent. Atmospheres containing known amounts of acetic anhydride were prepared and sampled through an absorber containing 20 ml of toluene, and then through a second absorber containing 10 ml of alkaline hydroxylamine reagent. The acetic anhydride in the first absorber was determined by adding 10 ml of hydroxylamine reagent and shaking the mixture for 5 minutes, after which a suitable portion of the lower layer was removed in a pipette; the volume of this portion was made up to 10 ml with alkaline hydroxylamine and 5 ml of acid ferric chloride reagent were added. The solution in the second absorber was treated similarly, and the optical densities of both solutions were measured. The two sets of figures are shown in graphs A and B of Fig. 3; the optical densities from the toluene absorber (curve A) have been divided by the fraction of the total aqueous phase taken for analysis. The acetic anhydride absorbed was calculated from these figures by means of curve D in Fig. 1; the recoveries are shown in Table III. From the slopes of the curves in Fig. 3, it is seen that

74·2 per cent. of the acetic anhydride is retained in the toluene and the remainder collected in the hydroxylamine.

TABLE III

ABSORPTION OF ACETIC ANHYDRIDE IN TOLUENE

Concentration of acetic anhydride in air, mg per cu. metre	Volume of air sampled, litres	Acetic anhydride absorbed		Calculated concentration of acetic anhydride, mg per cu. metre
		in toluene, mg	in hydroxylamine, mg	
10·3	90	0·760	0·200	10·7
15·0	90	1·02	0·345	15·2
21·7	90	1·37	0·500	20·8
22·6	90	1·54	0·586	23·6
32·7	90	1·76	0·735	27·7
42·7	90	2·64	0·946	39·8

A similar series of experiments was performed with keten, and the results are shown in Table IV; in calculating the keten from the hydroxylamine absorber, the optical density was applied to graph C in Fig. 1.

TABLE IV

ABSORPTION OF KETEN IN TOLUENE

Concentration of keten in air, mg per cu. metre	Volume of air sampled, litres	Keten absorbed		Calculated concentration of keten, mg per cu. metre
		in toluene, mg	in hydroxylamine, mg	
1·09	90	0	0·110	1·22
3·19	90	0	0·295	3·28
4·42	90	0	0·400	4·45
5·66	90	0	0·520	5·78

A series of atmospheres containing keten and acetic anhydride was prepared by diluting known amounts of acetic anhydride and the standardised keten solution with acetone and vaporising the solution in the air stream. The air was sampled through toluene and alkaline hydroxylamine and the colour developed as previously described. In order to calculate the recoveries, the reading from the toluene absorber was applied to graph A (Fig. 3) and the acetic anhydride content of the sample determined. The optical density corresponding to this amount of acetic anhydride was read on curve B of the same figure and this reading was subtracted from the optical density reading of the solution from the hydroxylamine absorber. The difference was applied to graph C in Fig. 1, whence the keten content of the sample was calculated. The results with the test atmospheres are shown in Table V.

TABLE V

ANALYSIS OF MIXED KETEN - ACETIC ANHYDRIDE ATMOSPHERES

Test atmospheres		Volume of air sampled, litres	Calculated concentration of	
keten, mg per cu. metre	acetic anhydride, mg per cu. metre		keten, mg per cu. metre	acetic anhydride, mg per cu. metre
1·83	11·8	90	1·92	10·2
1·83	22·7	90	1·49	20·0
1·83	32·2	90	2·12	27·6
1·83	43·5	90	1·76	38·9

DISCUSSION OF RESULTS

The method described may be regarded as satisfactory for the determination of acetic anhydride or keten alone in air. The determination of keten in the presence of acetic anhydride depends on the selective absorption of the latter in toluene and, as it has been shown that this is only about 75 per cent. efficient, the determination of keten in the presence of a large excess of acetic anhydride may be subject to serious experimental error. A formal

statement of the precision of the method would not be possible without extensive experimentation and a statistical analysis; the results in Table V show that no serious error is involved when the ratio of acetic anhydride to keten is of the order of 20 to 1, but it is doubtful if a greater ratio could be tolerated.

The efficiency of the absorption of acetic anhydride in toluene may depend to some extent on the rate of sampling and the design of the absorber. No other solvent has been found to give a more efficient absorption than toluene; silica gel and charcoal are not satisfactory.

If the amount of acetic anhydride present is much greater than the amount of keten, there may be a serious error in the estimation of keten if the percentage of acetic anhydride retained in the first absorber is not accurately known. This retention can be determined simply, and with sufficient accuracy, by assembling the toluene and alkaline hydroxylamine absorbers as described for the analysis of mixtures of acetic anhydride and keten, and connecting in front of them a bubbler containing a 0·1 per cent. v/v solution of acetic anhydride in toluene. Air is drawn through this system at the desired speed until 25 litres have been sampled. The contents of the two absorbers are analysed as described above, a 5-ml portion of the hydroxylamine extract of the toluene being used. If r_1 and r_2 are the optical densities produced by these two solutions, then the fractional absorption R in the toluene absorber is given by the expression: $R = 2r_1/(2r_1 + r_2)$. A number of absorbers of the type used in this investigation have been tested in this way and, in general, the value of R is higher than that found above, being about 0·8 to 0·9.

The fraction of the total keten retained in one hydroxylamine absorber can be determined experimentally if a pure keten atmosphere is available. If this is not possible, no serious error will be involved if this fraction is taken to be 0·8.

In order to calculate the keten and acetic anhydride content of an unknown atmosphere it is not necessary to prepare standard graphs from known atmospheres as described above. If a standard graph for ethyl acetate is prepared, the other graphs can be derived from it by taking into consideration the molecular weights and percentage reaction of keten and acetic anhydride with hydroxylamine, and from the measured absorption efficiency of the absorbers. As the standard graphs are linear for optical densities as high as 0·8, the keten and acetic anhydride content of an air sample are given by the following expressions—

$$\text{Keten content} = \left[\frac{0{\cdot}492}{S}m_2 - \frac{m_1}{\alpha}\left(\frac{1-R}{R}\right)\right],$$

$$\text{Acetic anhydride content} = \frac{1{\cdot}42}{\alpha R}m_1,$$

where m_1 and m_2 = ethyl acetate equivalents of optical densities of solutions from toluene and hydroxylamine absorbers, respectively,
α = fraction of toluene extract taken for analysis,
R = fraction of acetic anhydride retained in toluene
and S = fraction of keten retained in one hydroxylamine absorber.

References

1. Treon, J. F., Sigmon, H. E., Kitzmiller, K. V., Heyroth, F. F., Younker, W. J., and Cholak, J., *J. Ind. Hyg.*, 1949, **31**, 209.
2. Potts, A. M., Simon, F. P., and Gerard, R. W., *Arch. Biochem.*, 1949, **24**, 329.
3. Cameron, G. R., and Neuberger, A., *J. Path. Bact.*, 1937, **45**, 653.
4. Hestrin, S., *J. Biol. Chem.*, 1949, **180**, 249.
5. Williams, J. W., and Hurd, C. D., *J. Org. Chem.*, 1940, **5**, 122.
6. Gage, J. C., *J. Sci. Instr.*, 1953, **30**, 25.
7. ——, *Ibid.*, 1952, **29**, 409.

Imperial Chemical Industries Ltd.
Industrial Hygiene Research Laboratories
The Frythe, Welwyn, Herts.

January 5th, 1953

The Polarographic Determination of Iron and Chromium

By M. PERKINS and G. F. REYNOLDS

The development of a method for the simultaneous polarographic determination of iron and chromium in an alkaline mannitol base electrolyte is described.

Brief details of this method are presented; they include a calibration procedure for overcoming the slight mutual interference of the iron and chromium steps. Both sexavalent and tervalent chromium in solution are determined by making use of a bromate oxidation preceded and followed by a polarographic determination.

The method is satisfactory both in the presence and absence of nitrate ion, although the second of the two iron - mannitol complex waves is masked by the nitrate reduction step.

Approximate values for the stability constants of the ferri-mannitol and ferro-mannitol complexes are given, these values being calculated from the respective shifts of the half-wave potentials. Certain other theoretical aspects of the determination are also discussed.

Recently it became necessary to determine the concentrations of total iron and tervalent and sexavalent chromium in a large number of solutions, some of which contained nitrate ions. An investigation was accordingly undertaken to develop a polarographic method for determining these elements, if possible without a chemical separation procedure.

The work was carried out on a Cambridge photographic polarograph, but some determinations were also made with a linear-sweep cathode-ray polarograph[1,2] in order to show that this instrument, although not so readily available, could also be used with certain advantages.

Unless large concentrations of chloride are present, it is impossible to obtain reproducible polarographic steps for tervalent chromium, probably because of the irreversible nature of the reduction of the heavily aquated ion. However, sexavalent chromium in an alkaline base electrolyte is known to give an excellent step[3] from the reduction—

$$CrO_4'' + 4H_2O + 3e' \rightarrow Cr(OH)_3 + 5OH',$$

which occurs at a half-wave potential of $-0{\cdot}85$ volt *versus* the saturated calomel electrode. It appeared best, therefore, to determine the sexavalent chromium directly in alkali and the tervalent chromium after initially oxidising it to the sexavalent state.

Ferric iron cannot be directly polarographed ($Fe''' \rightarrow Fe''$; $E_0 = +0{\cdot}525$ volt *vs.* S.C.E.). Either it must be reduced to the ferrous state ($Fe'' \rightarrow Fe$; $E_0 = -0{\cdot}685$ volt *vs.* S.C.E.) or the tervalent state must be suitably complexed to change the half-wave potential to a more convenient value. The second alternative was preferable, as it avoided introducing a chemical reduction stage into the procedure. Also, as it had been previously decided to determine the chromium in an alkaline solution, a substance capable of complexing iron in such a medium was necessary.

Various complexing agents for ferric iron were tried, including triethanolamine,[4] alkaline tartrate[5] and mannitol.[6] The first gave a wave that interfered with the chromate step and the second failed to give easily reproducible results under the desired conditions. Mannitol, however, proved to be satisfactory. An alkaline mannitol supporting electrolyte was therefore adopted for the determination.

Experimental

Some unpublished work with alkaline mannitol, carried out for the Admiralty at a Naval Ordnance Inspection Laboratory, had led Winterton and Maber to conclude that the poor wave shape often obtained with alkaline solutions was due to "an electrical instability effect" and could be overcome by the use of a long drop-time. They reported that under these

conditions the shape of the wave was improved and there was "no steeply rising residual current curve." We therefore studied the reductions of both iron and chromium at dropping-mercury electrodes having drop-times ranging from 2·5 to 10 seconds. These drop-times were attained by the use of capillaries of various diameters while approximately the same pressure of mercury was retained. The results of these experiments are detailed below.

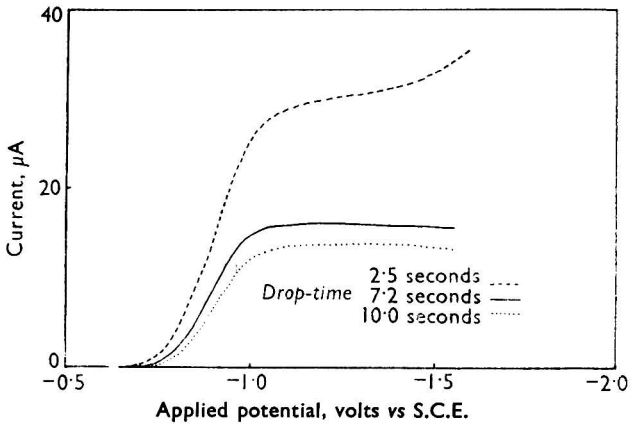


Fig. 1. Polarograms of 2×10^{-3} *M* chromate in 3 *M* potassium hydroxide solution

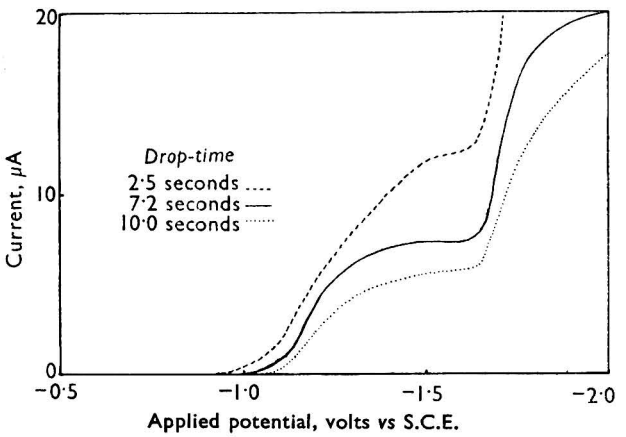


Fig. 2. Polarograms of 2×10^{-3} *M* ferric iron in 3 *M* potassium hydroxide solution

POLAROGRAPHY OF 2×10^{-3} *M* CHROMATE—

Fig. 1 shows polarograms of 2×10^{-3} *M* chromate in 3 *M* potassium hydroxide solution recorded with drop-times of 2·5, 7·2 and 10·0 seconds. The $m^{\frac{2}{3}}t^{\frac{1}{6}}$ values of these electrodes were 2·59, 2·10 and 1·51, respectively. Similar polarograms were recorded in *M* potassium hydroxide solution, but were less satisfactory in shape.

POLAROGRAPHY OF 2×10^{-3} *M* FERRIC IRON—

Fig. 2 shows polarograms of 2×10^{-3} *M* ferric iron in 3 *M* potassium hydroxide solution containing 0·2 *M* mannitol with drop-times of 2·5, 7·2 and 10·0 seconds. Similar polarograms recorded in *M* potassium hydroxide solution were again less satisfactory in shape.

DISCUSSION OF EXPERIMENTAL RESULTS—

It will be seen from Figs. 1 and 2 that both the chromium and the first of the two iron

steps are improved in shape when a 7-second drop-time is used instead of 2·5 seconds, although no further improvement appears to result from increasing the drop-time to 10 seconds. The use of a 3 *M* solution of potassium hydroxide instead of a *M* solution also improves the general shape of both the chromium and the iron steps. The reasons for this effect of drop-time and alkali concentration are not clear, although they may be associated with the surface conditions of the drop.

The chromium step, if the reduction is assumed to be represented by the equation—

$$CrO_4'' + 4H_2O + 3e' \rightarrow Cr(OH)_3 + 5OH',$$

occurs at an over-voltage of about 0·3 to 0·4 volt from the reversible voltage calculated thermodynamically. This over-voltage is almost certainly due to the irreversibility of the hydroxide formation at the electrode surface. The step, however, is remarkably well formed, and the limiting diffusion current is strictly proportional to the concentration of sexavalent chromium.

The occurrence of two waves in the ferri-mannitol polarogram shown in Fig. 2 indicates that the reduction is occurring in two stages—

(*a*) ferri-complex → ferro-complex;

(*b*) ferro-complex → iron.

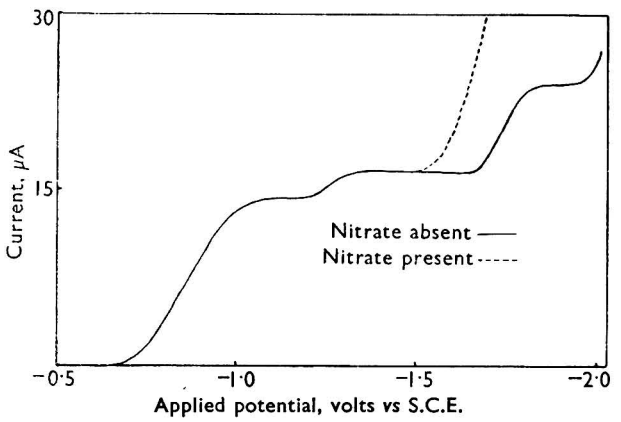


Fig. 3. Polarogram of a solution 2×10^{-3} *M* in respect of chromate and ferric iron in 3 *M* potassium hydroxide solution containing 0·2 *M* mannitol (drop-time, 7·2 seconds), in presence and absence of nitrate

This is confirmed by the ratio of the currents in the first and second stages of the reduction, which is about 1 to 1·8, compared with the theoretical value of 1 to 2. Also, the combined step heights of the ferric reduction (necessarily a 3-electron change) is within a few per cent. of the step arising from the reduction of sexavalent chromium to tervalent chromium of the same molarity. This is good evidence that both are 3-electron reductions, especially when the likely differences of the diffusion coefficients of such different molecules are considered.

From the data on half-wave potentials of the iron - mannitol complex reduction, approximate values for the stability constants of the complexes concerned can be evaluated. On the assumption of a half-wave potential of −1·385 volts for the reduction of uncomplexed ferrous ion and of values of −1·155 volts and −1·665 volts (all *vs*, S.C.E.) for the reduction of ferri-mannitol and ferro-mannitol complexes in *M* potassium hydroxide, and also that both complexes contain one Fe to one mannitol residue,[7,8] the constants $K_{Fe^{III}}$ and $K_{Fe^{II}}$ for the equilibria—

$$Fe^{III}\text{ - mannitol complex} + 3H_2O \rightarrow Fe''' + 3OH' + \text{mannitol}$$

and

$$Fe^{II}\text{ - mannitol complex} + 2H_2O \rightarrow Fe'' + 2OH' + \text{mannitol}$$

are calculated to be approximately 10^{-39} and 4×10^{-11}, respectively.

POLAROGRAPHY OF CHROMATE - FERRIC IRON MIXTURE—

Fig. 3 shows a polarogram of a solution 2×10^{-3} *M* with respect to both chromate and ferric iron in 3 *M* potassium hydroxide solution containing 0·2 *M* mannitol recorded with a dropping-mercury electrode of drop-time 7·2 seconds. It will be seen, as expected from the polarograms illustrated in Figs. 1 and 2, that the chromate and first iron steps have just sufficient separation to permit their measurement and that these two elements can, therefore, be determined in this solution if the drop-time is 6 to 7 seconds.

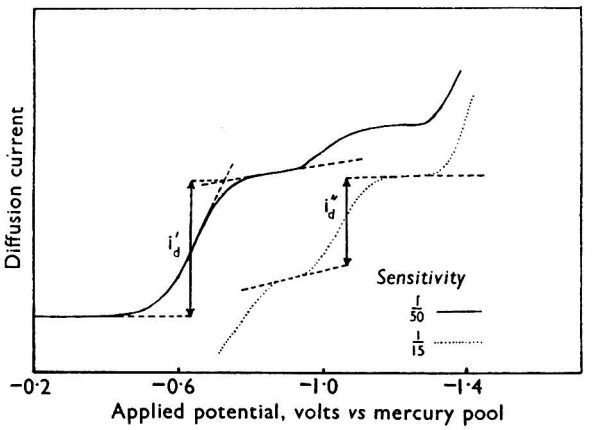


Fig. 4. Polarograms of 1×10^{-3} *M* chromate and 2×10^{-3} *M* ferric iron in 3 *M* potassium hydroxide solution containing mannitol

TABLE I

INTERFERENCE OF THE IRON STEP IN THE MEASUREMENT OF THE CHROMIUM STEP

All the solutions contained 112 μg of ferric iron per ml in the presence of 3 *M* potassium hydroxide and 0·2 *M* mannitol

Drop-time = 6·5 seconds

Chromium concentration, μg per ml	Step height,* divisions	Step height per μg per ml
80	180	2·25
72	162	2·25
64	143	2·24
40	88	2·21
16	34	2·13
8	11·5	1·45

* All step heights have been adjusted to their value at sensitivity 1/15 on the Cambridge polarograph used. At this sensitivity the constant iron concentration present in the solutions gave a step height of 47 divisions.

Because nitrate might be present in some of the solutions to be examined, the polarogram illustrated in Fig. 3 was repeated with nitrate added to the solution. Under these conditions a reduction wave attributed to the nitrate ion occurred at about −1·545 volts with respect to the saturated calomel electrode, and this interfered with the second iron step. The foot of this nitrate wave is shown in Fig. 3. The step is too large to be satisfactorily explained by any known reduction of the nitrate ion, and Meites[9] has recently stated that it is caused by the catalytic reduction of hydrogen ion.

CALIBRATION

Although in the absence of nitrate it is more accurate and convenient to measure the second (more sensitive) iron step, it was decided to specify measurement of the first step

for the purpose of routine analysis. This allowed the presence of nitrate, even in concentrations as high as 8 *M*, in the solutions under examination to be disregarded.

The chromium and the first iron steps are, as expected, so close together as to suffer slight mutual interference, the degree of which can be seen by reference to Tables I and II. In these determinations it is usual, unless the iron concentration is considerably greater than that of the chromium, to record the chromium and iron steps together and then to repeat the recording of the iron step on a higher sensitivity. A typical polarogram is shown in Fig. 4.

TABLE II

INTERFERENCE OF THE CHROMATE STEP IN THE MEASUREMENT OF THE IRON STEP

All solutions contained 64 μg of chromate per ml in the presence of 3 *M* potassium hydroxide and 0·2 *M* mannitol

Drop-time = 6·5 seconds

Iron concentration, μg per ml	Step height,* divisions	Step height per μg per ml	(Step height − 19) per (μg per ml − 80)
136	51·5	0·379	0·58
128	46·0	0·360	0·56
120	42·0	0·350	0·575
112	36·5	0·326	0·55
104	32·0	0·310	0·54
96	28·5	0·297	0·59
88	23·0	0·260	0·50
80	19·0	0·238	—
48	8·5	0·177	—
32	2·0	0·063	—

* All step heights have been adjusted to their value at sensitivity 1/10 on the Cambridge polarograph used. At this sensitivity the constant chromate concentration of 64 μg per ml gave a step height of 206 divisions.

It can be seen from Table I that the interference of the first iron step with the chromium step is slight, even when the iron concentration is considerably greater than that of the chromium. However, the step height of the chromium, although nearly proportional to its concentration, is not the same in the presence and absence of iron. It is therefore desirable to carry out the chromium calibration in the presence of an amount of iron approximately equal to the average iron concentration likely to be encountered.

TABLE III

VARIATION OF IRON STEP HEIGHT WITH VARYING CHROMIUM CONCENTRATION

All solutions contain 112 μg of ferric iron per ml in the presence of 3 *M* potassium hydroxide and 0·2 *M* mannitol

Drop-time = 6·5 seconds

Chromium concentration, μg per ml	Height of iron step,* divisions	(Step height − 24) per (96 − μg of Cr per ml)
96	24·0	—
88	28·0	—
80	29·5	0·345
72	33·0	0·375
64	34·5	0·330
56	37·0	0·325
48	39·0	0·315
40	41·0	0·305
32	45·0	0·328
24	47·5	0·327
16	50·5	0·330
0	55·5	0·328

* All step heights adjusted to sensitivity 1/10 on the Cambridge polarograph used.

The data in Table II show that, in the presence of a constant concentration of chromium, the height of the first iron step is not proportional to concentration, especially when the concentration is less than that of the chromate present. This is illustrated in the last column of Table II. A calibration curve is therefore necessary for converting the measured iron step heights to concentrations.

In addition to the non-linearity of the iron step height - concentration relationship, the amount of chromium present has a marked effect on the measured iron step height. This is shown in Table III, which also demonstrates that the step height expected from a given concentration of iron is a linear function of the concentration of chromium present.

CALCULATION OF THE IRON CONCENTRATION IN THE PRESENCE OF CHROMATE—

Because the iron step height varies linearly with chromium concentration, as shown in Table III, a simple expression relates the height of any iron step to the standard concentration of chromate, C_1, used in preparing a calibration graph as in Table II. This concentration, C_1, should be the approximate average concentration of chromium expected. For this a knowledge of the slope, K, of the curve relating the measured iron step height to the concentration of chromate, C_2, co-existing in the same solution, is also required.

The corrected value, D_2, of the measured iron step height, D_1, is then given by—

$$D_2 = D_1 - K(C_1 - C_2).$$

THE OXIDATION OF TERVALENT CHROMIUM

As an alkaline medium was to be used in the final polarographic procedure, an attempt was made to oxidise the tervalent chromium to the sexavalent state with alkaline peroxide. This oxidation, however, led to poor recoveries owing to the occlusion of chromic ions by ferric hydroxide. Bromate oxidation in acid solution was therefore finally adopted, as bromate and bromide in alkali do not interfere with either the iron or chromium polarographic reductions.

METHOD

DETERMINATION OF SEXAVALENT CHROMIUM—

Place in a 25-ml calibrated flask a suitable aliquot of the solution to be analysed, the volume taken being such that the final concentrations of the ions to be determined are within the range of concentrations used for calibration. Add 5 ml of *M* mannitol solution and 8 ml of 10 *M* potassium hydroxide, mix thoroughly and make up to volume with water. Transfer an aliquot to a polarographic cell, deoxygenate the solution for 5 minutes and record a polarogram at 25° C over the range −0·2 to −1·0 applied volts, at the "half voltage" setting of the polarograph (Note 1), using the mercury pool as anode and a dropping-mercury electrode with a drop-time of 6 to 7 seconds. Measure the height, H_1 (Note 2), of the step that occurs at about −0·6 volt.

Calculate the concentration of sexavalent chromium by comparison with standard solutions prepared as indicated above (Table I).

DETERMINATION OF TOTAL CHROMIUM AND IRON—

Take a second aliquot of the original solution and place it in a 100-ml tall-form beaker. Add 5 ml of water, 1 ml of *N* nitric acid and 0·5 g of potassium bromate. Evaporate in the presence of a "anti-bump" rod until only about 1 ml remains. Add a further 5 ml of water and repeat the evaporation. The odour of bromine should be absent at this stage; if not, the evaporation should be repeated.

Dilute with 5 ml of water and cool. Transfer to a 25-ml calibrated flask, add 5 ml of *M* mannitol and 8 ml of 10 *M* potassium hydroxide. Mix thoroughly and make up to volume with water. Transfer an aliquot to a polarographic cell, deoxygenate the solution for 5 minutes and record two polarograms at 25° C under the polarographic conditions described above. The first polarogram should be over a range of −0·2 to −1·0 applied volts and the second over a range of −0·8 to −1·4 applied volts (Note 3). Measure the height, H_2 (Note 2), of the chromium step that occurs at about −0·65 volt, and the height, D_1, of the iron step that occurs at about −1·05 volts.

Calculate the concentration of total chromium as before and calculate the concentration of iron with the aid of the calibration curves described under "Calibration" (Tables II and III).

NOTES—

1. The "half voltage" setting of the Cambridge polarograph applies only half the usual voltage to the main potentiometer. This has the effect of "stretching out" the steps over the record and making measurement easier.

2. Owing to the interference of the iron step with the top of the chromium step, results are more accurate if an "intersection method" of measurement is used. This is illustrated in Fig. 4.

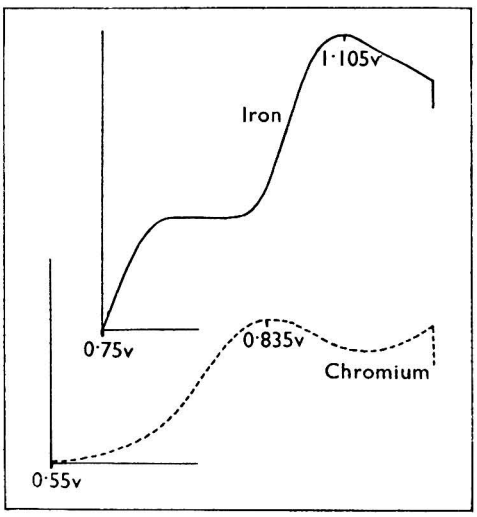


Fig. 5. Polarograms recorded on a cathode-ray polarograph for a solution 1×10^{-3} *M* in chromate and 2×10^{-3} *M* in ferric iron in 3 *M* potassium hydroxide solution containing mannitol.

Upper graph (solid line): iron wave; sensitivity, 1×10^3 ohms; starting potential, −0·75 volt.

Lower graph (broken line): chromium wave; sensitivity, 2×10^2 ohms; starting potential, − 0·55 volt

3. The separate recording of the two steps is generally necessary because the sensitivity setting required for the iron step is greater than that for chromium. If the concentration of iron is considerably greater than that of the chromium, the steps may be recorded at a single sensitivity.

ACCURACY AND REPRODUCIBILITY OF THE METHOD

To test the accuracy and reproducibility of the method a number of solutions containing various amounts of nitrate, iron and sexavalent and tervalent chromium were prepared and analysed. The results are shown in Table IV.

THE USE OF THE CATHODE-RAY POLAROGRAPH

Although the method described has been designed for use with a conventional instrument, it was desirable that it should also be satisfactory when used in conjunction with a linear-sweep cathode-ray polarograph of the type described in previous papers,[1,2] as instruments of this type have distinct advantages in speed of operation, associated with the direct reading of a continuously repeated trace. Experiments showed that a polarograph of this type gave

better separation of the chromium and first iron steps, with a consequent increase in accuracy and ease of measurement. In addition the rapidity of the polarographic determination was greatly increased. In this technique the waves for chromium and iron are obtained separately on the screen at start potentials of −0·55 and −0·75 volt, respectively. Typical waves are shown in Fig. 5.

TABLE IV

ACCURACY AND REPRODUCIBILITY OF THE METHOD

Nitrate concentration, *M*	Taken			Found		
	Chromium^{VI}, μg per ml	Chromium^{III}, μg per ml	Iron, μg per ml	Chromium^{VI}, μg per ml	Chromium^{III}, μg per ml	Iron, μg per ml
1	32	40	136	31	39·5	133
2	72	64	104	70	65·5	102
0	64	16	32	64	15	29
4	56	24	96	56·5	25	98·5
2	40	48	120	42	48	120
8	16	80	104	16·5	81	103·5
4	32	32	80	34	33	83
1	96	0	112	93·5	2	114·5
0	0	82	48	0	82	44

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

REFERENCES

1. Reynolds, G. F., and Davis, H. M., *Analyst*, 1953, **78**, 314.
2. Davis, H. M., and Seaborn, J. E., *Electronic Eng.*, 1953, **25**, in the press.
3. Lingane, J. J., and Kolthoff, I. M., *J. Amer. Chem. Soc.*, 1940, **62**, 852.
4. Jessop, C. T., *Nature*, 1946, **158**, 59.
5. Verdier, E. T., *Coll. Czech. Chem. Commun.*, 1939, **11**, 240.
6. Komarck, K., *Ibid.*, 1935, **7**, 404.
7. Traube, W., and Kuhbier, F., *Ber.*, 1932, **65**, 187.
8. ——, ——, *Ibid.*, 1933, **66**, 1545.
9. Meites, L., *J. Amer. Chem. Soc.*, 1951, **73**, 4115.
10. Harrison, T. S., and Storr, H., *Analyst*, 1949, **74**, 502.

CHEMICAL INSPECTORATE
MINISTRY OF SUPPLY
ROYAL ARSENAL
WOOLWICH, S.E.18

December 12th, 1952

The Potentiometric Determination of Quadrivalent Tellurium by Potassium Permanganate in Weakly Alkaline Solutions

BY I. M. ISSA AND S. A. AWAD

A procedure involving the potentiometric determination of quadrivalent tellurium with potassium permanganate in weakly alkaline solutions has been devised. The method is sensitive and is applicable to the determination of small amounts of tellurium^{IV}.

IN the course of determining the iso-electric point of tellurium dioxide (to be published elsewhere) by estimating its solubility in solutions of various pH values, it was deemed necessary, owing to the small solubility of the oxide, amounting to only 1 part in 150,000 parts of water, to devise a method suitable for the determination of small amounts of tellurium. The existing methods for determining quadrivalent tellurium include some that depend on

oxidation of quadrivalent tellurium to the sexavalent state in acid media by addition of a known excess of oxidising agents such as potassium dichromate,[1,2] potassium permanganate[3] or ceric sulphate[4] solutions, in which the excess of the oxidant is titrated with a standard solution of a suitable reductant. Other methods involve the use of an excess of a reducing agent, such as stannous chloride[2] or titanous chloride,[2] the excess being determined by the aid of a suitable oxidising substance. Some potentiometric methods have also been reported. These involve either reduction of tellurium^{IV} solutions with titanous chloride,[5] potassium iodide[6] or chromous chloride,[7] or oxidation with calcium hypochlorite.[8]

One of the methods previously applied involved the oxidation of tellurium^{IV} solutions in alkaline media with potassium permanganate[2]; the excess of oxidant was determined by standard oxalic acid solution after acidification of the solution.

The reduction of the permanganate ion in alkaline medium can be considered to take place in two sharply separated partial reactions[9]—

(*i*) the reduction of permanganate to manganate:

$$MnO_4' + e \rightarrow MnO_4''$$

(*ii*) the reduction of manganate to manganite:

$$MnO_4'' + 3H_2O + 2e \rightarrow H_2MnO_3 + 4OH'$$

Reaction (*i*) proceeds more rapidly than reaction (*ii*); the difference in their velocities increases with increasing hydroxyl-ion concentration. If, on the other hand, the alkalinity is decreased, the reduction to the manganite state is hastened, especially if an excess of the reductant is present. The separation of manganese dioxide will favour the reduction to the manganite state, and by the aid of its catalytic effect on the decomposition of potassium permanganate it will cause the velocity of the reaction to increase still further. As the redox potential of the MnO_4' - H_2MnO_3 system at unit hydroxyl-ion concentration is about 0·57 volt,[10] and that of the TeO_3'' - TeO_4'' system is about 0·4 volt[10] above that of the normal potential of hydrogen, it is possible to oxidise tellurite solutions quantitatively with potassium permanganate in an alkaline medium. From the E_0 values of the above two systems one can calculate the equilibrium constant K of the reaction—

$$2Mn^{\cdots\cdots\cdot} + 3Te^{\cdots\cdot} \rightarrow 2Mn^{\cdots\cdot} + 3Te^{\cdots\cdots\cdot},$$

and, hence, the degree of completion by the following relations—

$$\log K = \frac{E_1 - E_2}{0{\cdot}059/n}, \quad \alpha = K^{1/(z_1 + z_2)}$$

where E_1 and E_2 represent the standard redox potentials of the first and the second systems respectively, z_1 and z_2 the respective valency changes of both ion-pairs and n the number of Faradays that should be passed to complete the reaction. K amounts accordingly to $3{\cdot}5 \times 10^{-18}$ and α to $3{\cdot}1 \times 10^{-4}$, which indicate that the reaction can take place quantitatively. By using 0·66 volt[10] as the E_0 value for the system $Mn^{\cdots\cdots\cdot}/Mn^{\cdots\cdot}$, K becomes $3{\cdot}6 \times 10^{-27}$ and α 5×10^{-6}. Our aim was to find whether it is possible, by controlling the alkali concentration so that manganese dioxide only is formed, to follow the reaction potentiometrically.

Experimental

Solutions of quadrivalent tellurium were prepared by dissolving Scherring - Kahlbaum grade of sodium tellurite in pure water. The tellurium content of these solutions was determined by two different methods,[1,2] which gave concordant results. Potassium permanganate solutions were prepared from a commercial grade of the salt of high purity; all the necessary precautions were taken to guard against contamination from reducing organic matter. The solutions were filtered through sintered glass and were standardised with oxalic acid solution of known strength in acid medium. The oxalic acid solution used for standardising the potassium permanganate was prepared from a pure product containing not less than 99·8 per cent. of oxalic acid. This was standardised against 0·1335 *N* sodium hydroxide solution, with phenolphthalein as indicator. The sodium hydroxide solution was prepared by diluting a carbonate-free concentrated solution (about 18 *N*), kept in a waxed cylinder with carbon dioxide-free water in an atmosphere of nitrogen; it was stored out of contact with atmospheric carbon dioxide. The alkali content of the diluted solution was determined by titration with potassium hydrogen phthalate, phenolphthalein again being used as indicator. All the titrations were performed in an atmosphere of nitrogen. The potassium permanganate solution

was found to be 0·1650 N when standardised under these conditions, which permit its reduction to manganese^{II}. In the titrations described below, which were performed in dilute alkaline media, the reduction proceeds only to manganese^{IV}, when, accordingly, the normality amounts to 0·0990. The solution was kept in brown-coloured glass bottles, from which solutions at lower concentrations were prepared by accurate dilution.

A $9{\cdot}9 \times 10^{-3}$ N solution was prepared by diluting 25 ml of $9{\cdot}9 \times 10^{-2}$ N solution to 250 ml. A $9{\cdot}9 \times 10^{-4}$ N solution was prepared by diluting 10 ml of $9{\cdot}9 \times 10^{-2}$ N solution to 1000 ml. A $9{\cdot}9 \times 10^{-5}$ N solution was prepared by diluting 25 ml of the freshly prepared $9{\cdot}9 \times 10^{-4}$ N solution to 250 ml. The strength of the original stock solution was always checked before each dilution and was found to be constant. The water used in preparing and diluting the solutions was twice distilled from alkaline permanganate.

The concentration of the quadrivalent tellurium was checked by two methods.[1,2]

METHODS OF CHECKING CONCENTRATION OF TELLURIUM SOLUTION—

Method 1—Ten millilitres of the stock tellurite solution were mixed with 25 ml of 0·1650 N potassium permanganate solution and the solution was made 2 N in respect of sodium hydroxide. The mixture was heated and left for 30 minutes. The solution was then acidified with sulphuric acid, mixed with 50 ml of 0·0990 N oxalic acid solution, the excess of which was determined with 0·1650 N potassium permanganate. An average of 9·54 ml were necessary for complete oxidation of the excess of oxalic acid. From these results the normality of tellurium^{IV} solution was calculated to be 0·0749 N.

Method 2—Twenty millilitres of the tellurite solution were mixed with 20 ml of 0·1130 N potassium dichromate solution prepared from the twice recrystallised analytical reagent grade product that had been dried at 150° C,[11] and 5 ml of concentrated hydrochloric acid. The mixture was made up to 100 ml. After 30 minutes, 20 ml of 0·0954 N ferrous ammonium sulphate were added, and the excess of ferrous iron was estimated potentiometrically with 0·1130 N potassium dichromate solution. An average of 10·16 ml of potassium dichromate solution were needed for complete oxidation of the excess of ferrous iron. From these results the concentration of the tellurite solution was calculated to be 0·0750 N. Hence the mean value of the two concentrations of the tellurite solution amounted to 0·07495 N, which was considered for convenience as 0·075 N. From this tellurite solution others of lower concentrations were prepared in the same manner as the potassium permanganate solutions.

THE TITRATION DEVICE—

For the titration, an open 10-ml burette was used, which could be read to 0·02 ml. The titration vessel consisted of a tall-form well-steamed Pyrex-glass beaker, closed with a rubber stopper provided with appropriate holes. These served for the passage of the glass stirrer, the tip of the burette, the indicator electrode and the salt bridge. The stirrer was mechanically driven. The indicator electrode consisted of a platinum sheet 2 sq. cm in area, which was well cleaned, washed with water and ignited before use. The salt bridge, filled with saturated potassium chloride, served to connect the titration cell with the reference saturated calomel electrode.

RESULTS

The potentiometric titration of sodium tellurite solutions with potassium permanganate was always conducted in the cold. As these titrations were made in weakly alkaline medium, the solutions were made about 0·4 N in respect of sodium hydroxide. Under these conditions, with 0·075 N tellurium^{IV} and 0·099 N potassium permanganate solutions, the equilibria were quickly attained, 1 minute after each addition of the titrant. The titration graphs have steep inflections near the end-point amounting to 150 to 180 mV per 0·1 ml. Fig. 1 shows a representative titration graph under the foregoing conditions. The results by the usual volumetric methods,[1,2] 0·0749 N and 0·0750 N, were found to coincide closely with those obtained potentiometrically, 0·07494 N.

When the reacting solutions were diluted tenfold so that they were 0·0075 N in respect of tellurium^{IV} and 0·0099 N in respect of potassium permanganate, and the alkali concentration was kept at about 0·4 N, the titration failed to give quantitative results although the curves usually had steep inflections, which occurred after the equivalence point had been overstepped with an error of +4·0 per cent. At a higher concentration of alkali (about 0·8 N),

the error increased to +4·54 per cent. These higher results are probably due to the partial reduction of MnO_4' ion to MnO_4'' instead of to MnO_2, as the tellurium^{IV} concentration decreases. Titrations performed under these conditions were notable for the slow attainment of equilibria. These difficulties can, however, be overcome by increasing the rate of reaction (*ii*) (p. 488) by lowering the hydroxyl-ion concentration still more.

Attempts to carry out the titration without the addition of alkali, on the other hand, yielded results that were 1·4 per cent. lower than the theoretical value, but equilibria were attained rapidly.

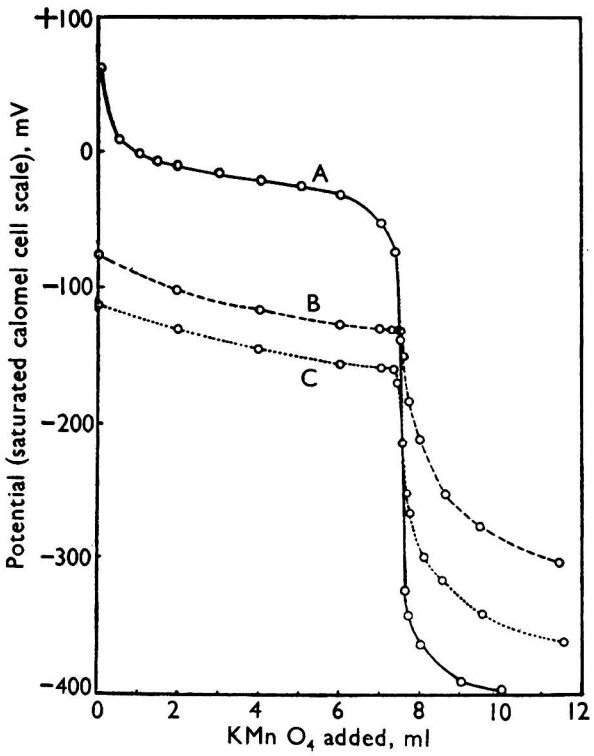


Fig. 1. Potentiometric titration of sodium tellurite with potassium permanganate. A, 0·075 *N* sodium tellurite solution and 0·099 *N* potassium permanganate solution; B, $7·5 \times 10^{-5}$ *N* sodium tellurite and $9·9 \times 10^{-5}$ *N* potassium permanganate; C, $7·5 \times 10^{-4}$ *N* sodium tellurite and $9·9 \times 10^{-4}$ *N* potassium permanganate

It was concluded therefore that the optimum alkali concentration, at which the most reliable result could be attained, lies at an alkalinity slightly higher than that of the tellurite solution. By varying the amount of alkali added, it was established that solutions about 0·08 *N* with respect to alkali gave the most accurate results. At this alkali concentration it was found possible to titrate more dilute solutions of quadrivalent tellurium with potassium permanganate. Fig. 1C, for example, represents a titration of $7·5 \times 10^{-4}$ *N* sodium tellurite with $9·9 \times 10^{-4}$ *N* potassium permanganate, whereas Fig. 1B represents a similar titration with these solutions diluted tenfold. When the tellurium solutions were further diluted to $7·5 \times 10^{-6}$ *N*, the titration failed to give an inflection at the end-point.

In all the above titrations, the curves were smooth with sharp inflections at the end-point. The magnitude of this inflection decreased with decrease in the concentrations of the reactants, and reached the minimum value of 23 mV per 0·1 ml of titrant when their normalities were $7·5 \times 10^{-5}$ in respect of sodium tellurite and $9·9 \times 10^{-5}$ in respect of potassium permanganate.

When the alkali concentration was regulated at the optimum value of about 0·08 *N*, equilibria were attained quickly after each addition of potassium permanganate solution.

Some representative results are shown in Table I.

TABLE I

EFFECT OF ALKALI CONCENTRATION ON RESULTS BY THE PROPOSED METHOD

Theoretical concentration of TeO_2, g per litre	Concentration of alkali, *N*	Concentration of TeO_2 from potentiometric titration, g per litre	Error, %
5·9854	0·4	5·9806	−0·08
0·5985	0·8	0·6257	+4·54
0·5985	0·4	0·6225	+4·00
0·5985	—	0·5890	−1·58
0·5985	0·08	0·5966	−0·31
0·05985	0·08	0·05961	−0·40
0·005985	0·08	0·005996	+0·18

From the foregoing results it can be seen that potassium permanganate oxidises quadrivalent tellurium in weakly alkaline solutions quantitatively at room temperature, without requiring precautions to be taken to exclude atmospheric oxygen. This method was applied to determining the iso-electric point of tellurium dioxide by estimating the tellurium content of buffer solutions saturated with the dioxide. The results obtained indicate that this point lies at pH 3·8, which is not far removed from the value of 4·2 given by Haïssinsky and Cottin[12] for the pH at which quantitative precipitation of tellurium dioxide takes place.

REFERENCES

1. Lenher, V., and Wakefield, H. F., *J. Amer. Chem. Soc.*, 1923, **45**, 1423.
2. Moser, L., and Miksch, R., *Monatsh. Chem.*, 1924, **44**, 349.
3. Schrenk, W. T., and Browning, B. L., *J. Amer. Chem. Soc.*, 1926, **48**, 2550.
4. Willard, H. H., and Young, P., *Ibid.*, 1930, **52**, 132.
5. Tomiček, O., *Bull. Soc. Chim. France*, 1927, **41**, 1389.
6. Someya, K., *Z. anorg. Chem.*, 1930, **187**, 357.
7. Lingane, J. J., and Niedrach, L., *J. Amer. Chem. Soc.*, 1948, **70**, 1997.
8. Tomiček, O., and Filipovic, P., *Coll. Trav. Chim. Tchécosl.*, 1938, **10**, 415.
9. Cited in Böttger, W., (Editor), "Newer Methods of Volumetric Analysis," Chapman & Hall Ltd., London, 1938, p. 40.
10. Latimer, W. H., "The Oxidation States of the Elements," Prentice-Hall, Inc., New York, 1938, pp. 79 and 223.
11. Furness, W., *Analyst*, 1950, **75**, 2.
12. Haïssinsky, M., and Cottin, M., *Anal. Chim. Acta*, 1949, **3**, 226.

CHEMISTRY DEPARTMENT
FACULTY OF SCIENCE
CAIRO UNIVERSITY
GIZA B, EGYPT

December 22nd, 1952

A Bromometric Method for the Determination of Formic Acid and Nitrite

BY MISS J. V. L. LONGSTAFF AND K. SINGER

A rapid method for the determination of formic acid is described. The method, which is also applicable to the determination of nitrite, is based on the oxidation of the formic acid by an excess of bromine in acetic acid, in the presence of pyridine, which acts as a catalyst, with subsequent iodometric titration of the residual bromine. The error in the determination of formic acid in 5 ml of 0·05 *M* solutions is less than 0·5 per cent.

A QUICK and accurate method for the determination of formic acid in the presence of nitric acid—and other acids—was required for the analysis of samples in work on the kinetics of

the oxidation of formic acid by nitric acid. The standard method of oxidation by alkaline permanganate[1] proved to be too slow and laborious, and other methods described in the literature, such as the gravimetric or volumetric method based on oxidation by mercuric chloride[2,3] and a colorimetric method,[4] were unsuitable for the same reason. An iodometric method based on oxidation by bromate or bromine appeared to be more convenient.

Preliminary experiments with aqueous bromate in acid solution showed that quantitative oxidation could be effected in approximately 30 minutes on a water-bath at 100° C, but that it was impossible to prevent loss of bromine under these conditions. Oberhauser and Hensinger[5] and Szelenyi[6] describe bromometric methods for determining formic acid. The former recommend the use of bromine in aqueous potassium bromide in the presence of sodium acetate or bicarbonate and the latter a solution of bromine and sodium acetate in acetic acid. It was found that the oxidation of formic acid when carried out under conditions similar to those described in these papers was slow. For example, the reaction between an excess of 0·1 *N* bromine solution in glacial acetic acid and formic acid either in the presence or absence of sodium acetate was incomplete after several hours at room temperature, whilst at higher temperatures the escape of bromine could not be prevented.

Hammick, Hutchison and Snell[7] have studied the rate of the reaction between bromine and formic acid in aqueous solution. They state that the oxidation is due to the reaction between formate ions and bromine molecules, and that the reaction is retarded by bromide and hydrogen ions, because of the formation of Br_3' ions and the suppression of the dissociation of formic acid.

It seemed that the rate of oxidation of formic acid by bromine might be increased by adding reagents that would combine with bromide ions, hydrogen ions or with both. The effect of addition of silver nitrate and of pyridine was investigated. Silver nitrate was added to remove the bromide ions and pyridine was used because of its basic properties and its known catalytic effect in bromination reactions.[8,9,10] With an approximately 100 per cent. excess of 0·1 *N* bromine in glacial acetic acid and silver nitrate the reaction was complete in 30 minutes at room temperature; with pyridine the reaction was complete in 2 minutes or less. Moreover, the addition of pyridine lowers the vapour pressure of bromine in glacial acetic acid and thus greatly reduces an otherwise considerable source of error.

In the method finally adopted, the oxidation is effected in 2 to 3 minutes by an approximately 100 per cent. excess of 0·1 *N* bromine solution in glacial acetic acid in the presence of 8 to 10 per cent. of pyridine. The residual bromine is determined by titration with thiosulphate after addition of potassium iodide. The strength of the bromine solution is found by a blank determination carried out simultaneously under the same conditions.

Method

Apparatus and reagents—

Flasks—Although loss of bromine is greatly reduced by pyridine, it is still occasionally noticeable when ordinary 500-ml conical flasks are used. The use of long-necked volumetric flasks eliminates this source of error completely. For the analysis of 5 or 10-ml samples of approximately 0·05 *M* formic acid 250-ml flasks are suitable.

Bromine solution—Make an approximately 0·1 *N* solution by dissolving 2·9 ml of bromine in a litre of glacial acetic acid. Add a small volume of water to prevent crystallisation of the acid, and store the solution in a glass-stoppered bottle. The composition of the solution changes slightly by evaporation of bromine into the space above it; this may lead to error, particularly when a series of determinations is carried out in which a large total volume of solution is used, as the volume of the gas phase into which evaporation can take place is considerably increased. For this reason the bromine solution is transferred, by means of a pipette, from a 500-ml calibrated flask, which is always kept more than half full. In this way the surface area of the solution and volume into which the bromine can evaporate remain relatively small.

The solution is transferred in an ordinary pipette into which it is sucked by means of a water pump. The high thermal expansion coefficient of acetic acid may lead to error if there is a marked temperature change during the period in which the bromine solution is transferred; this should not, in practice, cause any trouble and it is not necessary to take precautions.

Pyridine—Even analytical reagent grades of pyridine contain an impurity that consumes bromine at a measurable rate. This impurity is removed from good grades of pyridine by

distillation, in the presence of 1 per cent. v/v of bromine, under reduced pressure. Poorer grades are redistilled before purification with bromine. The purity of the pyridine is tested by carrying out three blank determinations (as described below) in which the bromine solution and pyridine remain together for 1, 3 and 5 minutes. If the titre figures for the three blanks agree, the pyridine is satisfactory. The effect of this method of purification on the pyridine is illustrated in Table I.

TABLE I

EFFECT OF PURITY OF PYRIDINE

Duration of reaction, minutes	Amount of 0·03948 *N* thiosulphate required in titration of unchanged bromine for a blank determination	
	With purified pyridine, ml	With unpurified pyridine, ml
1	31·43	33·34
3	31·35	33·12
5	31·38	32·64

Sodium thiosulphate solution—An approximately 0·1 *N* solution and a standard solution approximately 0·04 *N*.

Potassium iodide—A 20 per cent. solution of potassium iodide in water.

Starch solution.

PROCEDURE—

The amounts recommended are suitable for the determination of 10^{-4} to 5×10^{-4} moles of formic acid or nitrite. All solutions should be directed on to the bottom of the flask by means of a pipette, because any liquid remaining on the sides will not take part in the reaction.

Place approximately 2·5 ml of pyridine (by means of a graduated pipette) and the formic acid contained in 5 or 10 ml of aqueous solution in a 250-ml calibrated flask. Add from a pipette 20 ml of bromine solution. After 3 minutes, during which time the flask need not be stoppered, add 10 ml of potassium iodide solution and quickly rinse the sides of the flask with about 50 ml of distilled water. Add 5 ml of approximately 0·1 *N* thiosulphate solution from a pipette and titrate the residual iodine with standard 0·04 *N* thiosulphate in the usual way. The addition of 0·1 *N* thiosulphate (usually 5 or 10 ml, according to the strength of the formic acid solution) ensures a convenient titration figure.

Simultaneously carry out a blank determination, replacing the sample by an equal volume of water. The volume of standard thiosulphate equivalent to the formic acid in the sample is the difference between the titre of the blank and that of the sample.

Nitrite is determined in the same manner.

When a series of analyses is carried out, blank determinations should be made at the beginning and at the end of the series, as the strength of the bromine solution may vary slightly. The formic acid in eight solutions can be determined in about 1 hour.

NOTES—

1. Even purified pyridine is liable to react with bromine slowly; although this does not lead to any appreciable error with the recommended reaction times (*cf.* Table I), it may do so if reaction times are varied widely.

2. The reaction time of 3 minutes is ample to ensure quantitative oxidation. Usually the reaction is complete in less than 2 minutes, but the actual time required depends on the room temperature and on the amount of the excess of bromine. The reaction time can be further reduced by increasing the concentration of pyridine.

RESULTS

The agreement between the alkalimetric and the bromometric determination of 5 and 10-ml samples of an approximately 0·05 *M* formic acid solution is within 0·5 per cent. (Table II). Table II also shows the reproducibility of the results in a series of determinations

TABLE II

COMPARISON OF THE PROPOSED AND THE ALKALIMETRIC TITRATION METHODS FOR AN APPROXIMATELY 0·05 *M* SOLUTION OF FORMIC ACID

Amount of formic acid solution, ml	Formic acid found: By proposed method, g	Formic acid found: By alkalimetric titration, g
5	0·01057	
	0·01055	0·01061
	0·01059	
10	0·02117	
	0·02123	0·02122
	0·02120	

for samples of equal strength. The presence of nitrate does not affect the accuracy of the results (see Table III).

TABLE III

DETERMINATION OF FORMIC ACID IN PRESENCE OF NITRATE

Formic acid present, g	Sodium nitrate present, g	Formic acid found, g
0·01155	0·1008	0·01153
0·01155	0·1233	0·01154
0·01155	—	0·01159

The agreement between determinations of nitrite in a given solution by the bromometric method and by the standard chloramine-T method was as follows—

Sodium nitrite found, bromometric method, g	0·3264	0·3258
Sodium nitrite found, chloramine-T method, g	0·3252	0·3262

The authors wish to thank the Department of Scientific and Industrial Research for a grant to one of us (J. V. L. L.) and Imperial Chemical Industries Ltd. for financial assistance.

REFERENCES

1. Vogel, A. I., "Quantitative Inorganic Analysis," First Edition, Longmans, Green & Co., Ltd., London, 1939, p. 357.
2. Franzen, H., and Greve, G., *J. prakt. Chem.*, 1909, (2), **80**, 386.
3. Auerbach, F., and Plüddemann, W., *Chem. Zentr.*, 1909, *I*, 688.
4. Grant, W. M., *Anal. Chem.*, 1948, **20**, 267.
5. Oberhauser, F., and Hensinger, W., *Z. anorg. Chem.*, 1926, **160**, 366.
6. Szelenyi, G., *Z. Untersuch. Lebensmitt.*, 1932, **63**, 536.
7. Hammick, O. L., Hutchison, W. K., and Snell, F. R., *J. Chem. Soc.*, 1925, **127**, 2715.
8. Cross, W. E., and Cohen, J. B., *Chem. Soc. Pamphlet No.* 15, 1908.
9. Rosenmund, K. W., and Kuhnhenn, W., *Ber.*, 1923, **56***B*, 1262.
10. Kraus, E., *Ibid.*, 1923, **56***B*, 1801.

CHEMISTRY DEPARTMENT
ROYAL HOLLOWAY COLLEGE
ENGLEFIELD GREEN, SURREY

November 21st, 1952

Notes

THE COLORIMETRIC DETERMINATION OF SMALL AMOUNTS OF FLUORINE

MOST published methods for detecting and estimating ionic fluorine suffer from the disadvantage of depending on the diminution in intensity of colour of a reagent in the presence of fluorine. An exception to these, described by Feigl and Rajmann[1] has been applied to a detector paper method only.

It has now been found that when the thorium salt of 4-(*o*-arsonophenylazo)-N-(1-naphthyl)-ethylenediamine is prepared under strictly controlled conditions a brown colloidal dispersion is

formed, instead of the expected red precipitate; this brown form of the lake reacts with ionic fluorine in acid solution to give a bright red colour much more intense than the yellowish-brown of the reagent. The reaction is complete in a few minutes, which compares favourably with the several hours required by some published methods, *e.g.*, Lamar's.[2]

Method

Preparation of 4-(*o*-arsonophenylazo)-N-(1-naphthyl)-ethylenediamine—

Dissolve 1·08 g of B.D.H. *o*-aminophenylarsonic acid in 40 ml of water by warming. Add 10 ml of diluted analytical reagent grade hydrochloric acid (1 + 9) and then add ice to cool the mixture to between 0° and 3° C. Diazotise the solution at 0° to 3° C with 3·5 ml of a 10 per cent. sodium nitrite solution. The final volume should be about 75 ml. Destroy the excess of nitrite with sulphamic acid.

Add the diazotised solution to a solution, cooled with ice to 0° C, of 1·3 g of B.D.H. N-(1-naphthyl)-ethylenediamine dihydrochloride prepared in 50 ml of warm water. The final volume for coupling should be 200 ml and the temperature between 0° and 3° C. The solution should be acid to Congo red. Stir for 1 hour and filter. The product is not washed or dried. Determine the moisture content of the pressed cake; the dry yield should be about 2·2 g. Make the paste up with distilled water to such a volume as to give a concentration of 1 g of dry dye per litre. This is the dye suspension. This dye suspension is stable; it can be stored on a laboratory shelf for at least 4 months without change.

Reagents—

Lake dispersion—Add 3·0 ml of diluted hydrochloric acid (1 + 9) to 100·0 ml of the 0·1 per cent. dye suspension. Add 3·0 ml of a solution of 6·96 g of thorium nitrate tetrahydrate (B.D.H. laboratory grade) in 20 ml of water and make up to 1 litre with distilled water. It should be kept for 3 hours before use, and will remain in good condition for at least 3 days if stored in the dark at room temperature.

Sodium chloroacetate buffer solution[2]—Dissolve 94·45 g of chloroacetic acid (B.D.H. laboratory grade) in 400 ml of cold water and add a solution of 30·0 g of AnalaR sodium hydroxide in 250 ml of water. Keeping the temperature below 30° C, make the solution up to 1 litre with water.

Procedure—

Small quantities of fluorine are most conveniently estimated by visual comparison of the colour given by an unknown with that given by a series of standards. Results are more accurate when comparisons are made with a short range of standards made from sodium fluoride solutions in which the amount of lake dispersion added is not greatly in excess of that completely used up by the highest concentration in the range. Convenient ranges are as follows—

For 0 to 0·6 μg per ml of fluorine in 0·1-μg steps, use 0·5 ml of lake dispersion in 100 ml.
For 0·6 to 1·5 μg per ml of fluorine in 0·2-μg steps, use 1·5 ml of lake dispersion in 100 ml.
For 1·5 to 2·7 μg per ml of fluorine in 0·3-μg steps, use 3·0 ml of lake dispersion in 100 ml.

Place 100 ml of the test solution in a 100-ml Nessler glass, add 0·5 ml of chloroacetate buffer (pH is then about 3·5) and 0·5 to 3·0 ml of lake dispersion, according to the range required. Set the mixture aside for 3 minutes and compare the colours in a white light, preferably north daylight, with a range of standards containing convenient amounts of fluorine. The colour differences given by less than 0·1 μg of fluorine per ml are better observed if the test solutions are left to stand for up to 20 minutes. Up to 7 ml of buffer may be used if required.

A standard method[3] of isolating fluorine by distillation is advised when the sample is dirty or when large quantities of interfering ions are present.

Interfering substances—

A few common substances likely to upset the determinations have been examined and their interference with the estimation of a solution of ionic fluorine containing 0·2 μg per ml is recorded in Table I. A plus sign indicates that the colour is intensified and consequently the fluorine found is too high by the amount shown; a minus sign indicates the reverse.

Free chlorine destroys the reagent, but can be removed by treatment with a slight excess of hydroxylamine hydrochloride.

TABLE I

EFFECT OF INTERFERING SUBSTANCES ON FLUORINE DETERMINATIONS

Interfering compound	Amount, μg per ml	Effect, μg per ml
Di-sodium hydrogen phosphate	2	+0·15
Sodium borate	10	none
Sodium arsenate	5	+0·05
Sodium sulphate	100	−0·1
Sodium chloride	100	+0·1
Aluminium chloride	2	−0·1
Magnesium chloride	10	+0·05
Calcium chloride	100	none
Sodium nitrate	200	−0·1

The author's thanks are due to the Chief Scientist, Ministry of Supply, for permission to publish this note, and to Miss S. Williams who has carried out much of the analytical work.

REFERENCES

1. Feigl, F., and Rajmann, E., *Mikrochemie*, 1932, **12**, 133.
2. Lamar, W. L., *Ind. Eng. Chem., Anal. Ed.*, 1945, **17**, 148.
3. Sanchis, J. M., *J. Amer. Wat. Wks. Ass.*, 1936, **28**, 1456.

MINISTRY OF SUPPLY
CHEMICAL DEFENCE EXPERIMENTAL ESTABLISHMENT
PORTON, WILTSHIRE

H. F. LIDDELL
January 9th, 1953

THE *p*-HYDROXYDIPHENYL TEST FOR ACETALDEHYDE: THE DETERMINATION OF ALANINE AND THREONINE IN PROTEIN HYDROLYSATES

p-HYDROXYDIPHENYL was first used for the detection of acetaldehyde by Eegriwe.[1] A quantitative colorimetric determination based on this reaction was developed by Miller and Muntz[2] and has found wide application in biochemical work for the determination of acetaldehyde and of substances, such as lactic acid, alanine and threonine, that can be readily oxidised to acetaldehyde. The original method of Miller and Muntz was much improved by Barker and Summerson,[3] who found that colour formation was catalysed by copper salts and who introduced the use of an alkaline solution of the *p*-hydroxydiphenyl in place of the solid reagent.

In this laboratory the reaction has been used for determining alanine and threonine in protein hydrolysates. It was found that the procedure suggested by Barker and Summerson[3] and later modifications by other workers[4,5] failed to give reproducible results. A quantitative study of the reaction showed that the colour intensity reached a maximum and subsequently declined, which indicated the occurrence of opposed reactions of colour formation and destruction. High temperatures and high concentrations of sulphuric acid favoured destruction, and it is interesting that widely different conditions have been used by different workers; temperatures of 0° to 37° C for colour development and concentrations of 84 to 98 per cent. v/v of sulphuric acid have been reported in the literature.[2,3,4,5,6]

While the sulphuric acid concentration and the temperature used for colour development can be readily controlled, comparatively high temperatures occur on two occasions during the usual procedure. The first is when the alkaline reagent is added to the strongly acid solution of acetaldehyde, itself already warm through dilution. Stotz[5] recommended carrying out this part of the procedure in a bath of ice, and this has been found in this laboratory to assist greatly in reducing the variability in colour intensity. The second occasion is when, at the end of colour development, the tubes of reaction mixture are immersed in boiling water for 90 seconds to remove undissolved reagent. It has been found that if colour development is allowed to proceed for a longer time the excess of reagent dissolves without heating and a much greater colour intensity results.

MODIFIED METHOD

REAGENTS—

Sulphuric acid—Analytical reagent quality, sp.gr. 1·84, 98 per cent. w/w; see also next section.

Copper sulphate—A 4 per cent. w/v solution of $CuSO_4.5H_2O$.

p-*Hydroxydiphenyl*—A 1·5 per cent. solution of the B.D.H. preparation in 0·5 per cent. sodium hydroxide.

PROCEDURE—

Transfer 2 ml of the acetaldehyde solution by pipette into a thin-walled test tube, 23 × 150 mm, add 0·1 ml (2 drops) of copper sulphate and cool the tube by placing it in a bath of ice at 0° to 5° C for at least 5 minutes. Then add 10 ml of sulphuric acid; this addition should take at least 30 seconds and the tube should be well shaken throughout this operation. Replace the tube in the bath of ice for a further 5 to 10 minutes. Then add 0·2 ml (5 drops) of *p*-hydroxydiphenyl reagent with thorough mixing after the addition of each drop. Return the tube to the bath of ice for 5 minutes and then transfer it to a bath of water at 27° ± 1° C. Shake to re-disperse the reagent at half-hourly intervals. Determine the colour intensity after 2½ hours with a Spekker absorptiometer, 1-cm cells and an Ilford No. 605 yellow-green filter, using a reagent blank made up with distilled water as the reference standard.

The acetaldehyde solution should contain 1 to 8 μg of acetaldehyde per millilitre, and should be free from other aldehydes and from nitrates and nitrites; results are unaffected by the presence of sodium bisulphite in concentrations up to 1 per cent. The colour intensity remains unchanged if development is continued for a further 60 minutes.

Determinations of standard acetaldehyde solutions on different days gave absorptiometer readings with a maximum variation of 2 per cent. It is desirable, however, to carry out daily determinations at one or two levels of acetaldehyde as a check on the standardisation.

If more rapid development of colour is required, the test can be performed with 1 ml of acetaldehyde solution (1·5 to 10 μg of acetaldehyde) and quantities of other reagents as above, but 0·4 ml of *p*-hydroxydiphenyl reagent should be used. Develop the colour at 27° C for 70 minutes. Results are slightly more variable at this acid concentration and can be improved by longer development at lower temperatures.

EFFECT OF IMPURITIES IN THE SULPHURIC ACID

Some batches of sulphuric acid of analytical reagent grade have been found to give discoloured blanks with this method, and to cause insensitivity of the method at low levels of acetaldehyde (up to 4 μg), with poor colour formation at higher levels. Similar effects have been reported[7] to be caused by traces of nitrates and nitrites in the sulphuric acid. All unsatisfactory batches of analytical reagent grade acid examined in this laboratory have been found to give a deep blue colour with diphenylamine.[8] While sulphuric acid that passes this test for "nitrate" should be satisfactory, and all such batches tested have been found to be, it is conceivable that other substances, not detected by the diphenylamine test, might also interfere with the acetaldehyde reaction. It is therefore recommended that before use each new batch of acid should be tested for its suitability with small amounts of acetaldehyde; under good conditions the calibration curve is almost linear and shows no point of inflection at low acetaldehyde concentrations.

DETERMINATION OF ALANINE

Alanine was determined by a modification of the method of Virtanen and Rautanen.[9] A 2-ml sample of a protein hydrolysate, prepared by the method of Henderson and Snell[10] and containing 50 to 200 μg of alanine per ml, was heated under reflux with 2 ml of a 2 per cent. solution of ninhydrin and 10 ml of a solution containing 10 per cent. of potassium dihydrogen phosphate and 21 per cent. of sodium chloride, the acetaldehyde produced being aspirated into 10 ml of a 1 per cent. solution of sodium bisulphite. After 65 minutes the bisulphite solution was diluted to 25 ml and 2-ml aliquots were taken for the acetaldehyde determination. Parallel determinations of standard alanine solutions must be performed, as only 95 to 97 per cent. recoveries of acetaldehyde are attained under these conditions.

Alexander and Seligman[4] reported that aldehydes from other amino-acids also gave colours with *p*-hydroxydiphenyl, although this effect was less when the colour was developed at 37° C. In view of the variability of the colour reaction at high temperatures, it was decided to use the procedure described above and to correct for the effect of other amino-acids. Determinations with other amino-acids showed that only four of those normally found in proteins interfered appreciably. The corrections to be applied are shown in Table I together with those reported by Alexander and Seligman. These corrections are expressed as the number of equivalents of alanine that would give the same colour intensity as 100 equivalents of the interfering amino-acid. Thus if, for example, 5·0 per cent. of the total nitrogen of a protein hydrolysate is aspartic acid nitrogen, then a correction of (5·0 × 4)/100 must be made in the value of apparent alanine nitrogen, *i.e.*, 0·2 must be subtracted as a correction for the aspartic acid also present.

TABLE I

CORRECTIONS FOR OTHER AMINO-ACIDS IN THE ESTIMATION OF ALANINE

(Expressed as the percentage of amino-acid nitrogen detected as apparent alanine nitrogen)

Amino-acid	Proposed method	Alexander and Seligman
Aspartic acid	4	4
Leucine	13·5	9 to 20
*iso*Leucine	4	No effect
Glycine	4	Blue green colour not detected with Klett 540 filter
Norleucine	Not investigated	20 to 30
Norvaline	Not investigated	30

Tests on a number of proteins gave results in which the maximum variation between duplicate determinations was 3·6 per cent. and was, for some proteins, as low as 0·3 per cent.

By the use of only 2 ml of sodium bisulphite for absorption, and then making this solution up to 5 ml and using 2-ml aliquots for the colour reaction, it has been found possible to determine reliably as little as 20 μg of alanine in yeast extracts.

DETERMINATION OF THREONINE

A modification of the method of Winnick,[11] making use of Conway micro-diffusion units, was used for the determination of threonine. A protein hydrolysate as previously described was diluted (1 + 2) so as to contain 15 to 60 μg of threonine per millilitre, and 1 ml was transferred by pipette to the outer chamber of a Conway unit. Oxidation was by periodic acid in the presence of phosphate buffer as in the standard method, and the acetaldehyde was allowed to diffuse for at least 5 hours into 1 ml of a 1 per cent. solution of sodium bisulphite in the central chamber. A 0·5-ml portion of this solution, diluted to 2 ml or 1 ml with distilled water, was used for the colorimetric determination of acetaldehyde by the procedures described above. Determinations of standard solutions containing 30 and 60 μg of DL-threonine per millilitre were used to construct a standard curve.

Determinations of threonine in a series of proteins by the rapid (1 ml of solution) procedure for acetaldehyde showed a maximum variation between replicates of 13·6 per cent., although the variation was as low as 0·3 per cent. for some proteins. Determinations of threonine in specimens of gliadin and edestin, kindly given by Professor A. C. Chibnall, gave values of threonine nitrogen of 1·39 and 2·39, respectively, the extreme range of the estimations being 1·30 to 1·49 and 2·32 to 2·50. Rees[12] reported values of 1·40 and 2·43 for the same protein preparations by his macro-method of periodate oxidation followed by iodimetric titration.

The author wishes to express his thanks to Dr. Anne S. Cole for directing his attention to the problem of poor colour formation with some sulphuric acids and for helpful discussion; and to Dr. E. W. Yemm for encouragement and advice at all stages of the work.

REFERENCES

1. Eegriwe, E., *Z. anal. Chem.*, 1933, **95**, 323.
2. Miller, B. F., and Muntz, J. A., *J. Biol. Chem.*, 1938, **126**, 413.
3. Barker, S. B., and Summerson, W. H., *Ibid.*, 1941, **138**, 535.
4. Alexander, B., and Seligman, A. M., *Ibid.*, 1945, **159**, 9.
5. Stotz, E., *Ibid.*, 1943, **148**, 585.
6. Block, R. J., and Bolling, D., *Ibid.*, 1939, **130**, 365.
7. Russell, J. A., *Ibid.*, 1944, **156**, 463.
8. "AnalaR Standards for Laboratory Chemicals," Second Edition, British Drug Houses Ltd. and Hopkin & Williams Ltd., London, 1937, p. 266.
9. Virtanen, A. I., and Rautanen, N., *Biochem. J.*, 1947, **41**, 101.
10. Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, 1948, **172**, 15.
11. Winnick, T., *Ibid.*, 1942, **142**, 461.
12. Rees, M. W., *Biochem. J.*, 1946, **40**, 632.

DEPARTMENT OF BOTANY
THE UNIVERSITY
BRISTOL, 8

B. F. FOLKES
December 23rd, 1952

THE CHARACTERISATION OF VINEGAR BY ITS ALBUMINOID AMMONIA VALUE

In the examination of vinegars the determination of total nitrogen is useful in detecting sophistication with artificial products. It has been observed that there exists a broad correlation between the figures for total nitrogen and the albuminoid ammoniacal nitrogen. Determination of the latter is far more rapid and less tedious than that of total nitrogen, and has been found to be a valuable routine sorting test.

Method

Reagents—

Sodium hydroxide solution, 0·2 N.

Alkaline potassium permanganate solution—Prepare this and the following reagent according to Thresh, Beale and Suckling.[1]

Nessler reagent.

Standard ammonium chloride solution—Prepare a stock solution by dissolving 3·82 g of ammonium chloride crystals, A.R., dried at 105° C, in distilled water, and making up to 1 litre. Dilute 1 ml of this stock solution to 100 ml with distilled water. Each millilitre of the dilute solution contains 0·01 mg of nitrogen.

Procedure—

Neutralise 5 ml of sample with 0·2 *N* sodium hydroxide solution, dilute to 100 ml in a calibrated flask with ammonia-free distilled water (which is used throughout) and mix. Transfer 20 ml by pipette into a distillation flask, add 230 ml of water, a pinch of sodium carbonate and a few pieces of recently ignited pumice to prevent bumping, and distil until 100 ml have been collected. Reject this distillate. Add 50 ml of alkaline potassium permanganate reagent and 100 ml of water to the liquid left in the distillation flask and again distil until exactly 100 ml have been collected. Determine the ammonia in this second distillate by the usual Nessler method, as follows. Take an aliquot (10 ml is suitable for malt vinegars), dilute with water to 50 ml in a Nessler cylinder, add 2 ml of Nessler reagent and mix. Match the colour after 5 minutes against dilutions prepared in a similar manner from the standard ammonium chloride solution.

If a represents the number of ml of standard ammonium chloride solution required for matching and b the number of ml of distillate taken for Nesslerising, then—

Albuminoid ammoniacal nitrogen in the sample $= a/b \times 1000$ p.p.m.

Results

By the procedure described the following figures for albuminoid ammoniacal nitrogen in genuine malt vinegars were found—

Total nitrogen, p.p.m. (w/v) ..	820	810	460	440	840	600	750
Albuminoid ammoniacal nitrogen, p.p.m. (w/v)	380	375	230	208	400	320	350

The corresponding figures for eight artificial vinegars were: total nitrogen, 11 to 34 p.p.m., and albuminoid nitrogen, nil to 4·0 p.p.m. The wide difference between the albuminoid ammonia figure for genuine malt vinegars and for artificial products is at once evident.

This method of examination, which has so far been restricted to the analysis of water, has been applied for the first time to the analysis of foodstuffs. The method is being extended to the examination of soft drinks, such as squashes, cordials and fruit juices, and encouraging results have already been obtained.

The author thanks Mr. J. M. Roy for his help.

Reference

1. Taylor, E. W., "The Examination of Waters and Water Supplies (Thresh, Beale and Suckling)," Sixth Edition, J. and A. Churchill, Ltd., London, 1949, p. 755.

West Bengal Public Health Laboratory
School of Tropical Medicine
Calcutta, 12, India

S. N. Mitra
December 22nd, 1952

THE DECOMPOSITION OF AMMONIA IN SEALED-TUBE MICRO-KJELDAHL DIGESTIONS WITH A SELENIUM CATALYST

GRUNBAUM, Schaffer and Kirk,[1] by the method of White and Long,[2] but without catalyst, have shown that when digestion is carried out above 450° C the recovery of ammonia from ammonium sulphate is low, and approaches zero at 550° C. They indicated that this decomposition was delayed in the presence of organic matter, no loss resulting from 30 minutes digestion at 470° C.

Before the publication of their results we had found a similar loss of ammonia when using a mercury catalyst, as recommended by White and Long.[2] In view of the adverse reports of several workers, such as Willits, Coe and Ogg,[3] on the use of selenium, we investigated the effect of a mercury - selenium catalyst under these conditions. We have found that the maximum temperature for quantitative recovery is only about 420° C if such a catalyst is used. The experimental details were as suggested by White and Long,[2] except that the catalyst was a mixture of 9 g of mercuric sulphate and 2 g of selenium, about 20 mg being used for each determination. A vertical Carius-type furnace was used and the temperature was measured both with a calibrated thermocouple and with a mercury thermometer (up to 500° C). The samples were digested for 45 minutes because of erratic preliminary results, which are now known to have been caused by insufficient temperature control.

Typical results for three pure test substances used in this laboratory are shown in Table I.

TABLE I

EFFECT OF TEMPERATURE ON RECOVERY OF NITROGEN

Temperature, ° C	Nitrogen found in amino-acetic acid, %	sulphanilic acid, %	4-amino-pteridine, %
360	18·6	8·1	47·8
400	18·7	—	47·6
420	18·6	8·0	47·7
440	18·1	---	46·5
460	17·6	7·4	42·9
500	14·1	5·3	35·8
550	nil	nil	—
Theoretical amount of nitrogen—	18·7	8·1	47·6

Grunbaum, Schaffer and Kirk[1] suggested that the temperature is a more critical factor than the time of digestion. This is confirmed by the results, shown in Table II, of prolonged digestions at 420° and 465° C, with a mercury - selenium and mercury catalyst, respectively.

TABLE II

EFFECT OF DIGESTION TIME ON RECOVERY OF NITROGEN FROM AMINO-ACETIC ACID

Time, hours	Nitrogen found at 420° C, %	465° C, %
¾	18·6	18·7
2	18·8	—
3	18·4	18·45
4	—	18·5
Theoretical amount of nitrogen—	18·7 per cent.	

Selenium in sealed-tube digestions thus appears to lower, by about 50° C, the temperature at which decomposition of the ammonia occurs. Lake, McCutchan, van Meter and Neel[4] have shown that decomposition of ammonia occurs in open tubes above 410° C in the absence of selenium, and addition of selenium to such a digestion should by analogy result in loss of ammonia if the temperature approaches 360° C, which, from the graph given by the same authors,[4] is the boiling point, at a pressure of 760 mm of mercury, of a solution containing 0·5 g per ml of potassium sulphate in sulphuric acid. The work of Willits, Coe and Ogg[3] indicates that the amount of ammonia lost owing to selenium is dependent on the potassium sulphate concentration, and hence on the temperature attained. It is also apparent from the work of these authors,[3] and of Patel and Screenivasan,[5]

that decomposition of the ammonia does not occur until decomposition of the sample is complete. These findings probably explain the successful use of selenium by many workers, who have either used insufficient potassium sulphate to raise the temperature to that required for decomposition of the ammonia, or have digested for no longer than the minimum time necessary for complete recovery.

The author wishes to thank Miss O. Pugh for technical assistance.

REFERENCES

1. Grunbaum, B. W., Schaffer, F. L., and Kirk, P. L., *Anal. Chem.*, 1952, **24**, 1487.
2. White, L. M., and Long, M. C., *Ibid.*, 1951, **23**, 363.
3. Willits, C. O., Coe, R. R., and Ogg, C. L., *J. Ass. Off. Agric. Chem.*, 1949, **32**, 118.
4. Lake, G. R., McCutchan, P., van Meter, R., and Neel, J. C., *Anal. Chem.*, 1951, **23**, 1634.
5. Patel, S. M., and Screenivasan, A., *Ibid.*, 1948, **20**, 63.

CHEMICAL DIVISION
WELLCOME RESEARCH LABORATORIES
BECKENHAM, KENT

P. R. W. BAKER
December 17th, 1952

Apparatus

A DOUBLE SYRINGE-PIPETTE FOR DISSOLVED OXYGEN ESTIMATIONS

THE classical method of Winkler is widely used in the determination of dissolved oxygen in a variety of natural waters. Samples should always be treated with the reagents as soon as possible after sampling since, with waters saturated or super-saturated with oxygen and with the air at a temperature higher than the water, expulsion of gas may take place; even with unsaturated samples the dissolved oxygen content may change on standing, as a result of biological processes. Consequently the reagents must often be added at the place where the samples are taken. Frequently in such field-work and particularly in investigations of depth profiles in the sea, for which purpose the apparatus described here was designed, samples are taken that all require addition of the reagent within a relatively short time. It is common procedure to deliver the sample for analysis direct from the collecting device into calibrated bottles having a capacity of 100 to 200 ml. The manganese sulphate and potassium iodide - potassium hydroxide reagents are then added successively from graduated pipettes, delivery being made at the bottom of the bottle. The bottle is stoppered and shaken and, after the precipitate has been allowed to settle, acid is added to liberate the iodine. The whole of the sample is titrated and allowance is made for the amount of water expelled by the addition of the reagents. This is the standard technique in sea-water analysis. The use of two pipettes in this way with two separate reagent bottles is inconvenient and, in bad sea conditions, not conducive to accuracy. To overcome this difficulty a simple double-pipette has been devised which delivers the two solutions simultaneously; pipettes and reagent bottles are not necessary.

THE DOUBLE SYRINGE-PIPETTE

A simple pipette was designed, which delivered simultaneously successive 1-ml samples of each of two reagents (manganous sulphate solution and a mixture of potassium iodide and potassium hydroxide solutions). The apparatus has been made largely from Perspex, but if facilities were available for machining, other materials could, with advantage, be used. For indoor work only, glass joints can replace some of the rubber connections, but for work at sea it is desirable to have rubber connections, which can be more easily replaced in the event of an accident.

Two ordinary 15-ml hypodermic syringes, A, are mounted in a square frame of Perspex, B; the bottom of the syringes pass through the lower cross bar, C, of the frame. The heads of the syringes, D, are fastened to a moving cross-piece, E, with flanges, F, which run in a slot, G, cut out of the sides, H, of the square Perspex frame. The heads of these two syringes are fastened via this cross-piece to a large $\frac{3}{8}$-inch Whitworth screw, N. A large milled nut, J, held in position by the cross-plate, K, which is mounted on the cross bars, L, on the top of the frame, enables the plungers to be raised or lowered a given distance by making a constant number of turns. A zero mark is made on the knurled nut, J, and on the fixed brass plate, K; three turns of J, with the particular syringes we used, were found to deliver about 1 ml.

The whole of the square frame is backed with a solid piece of Perspex, M, and is clamped on to a stand. The lower ends of the two pipettes are connected by rubber tubing, Q, to two

two-way taps (with capillary bore), P. The inside legs of the other side of each tap are taken to the two legs of the capillary delivery tubes, R. These are drawn out to deliver small drops of solution and their inside faces at S are ground flat. The flat surface helps to reduce the size of their cross-section; this is necessary because oxygen bottles are designed with narrow necks, and, in addition, it also helps to keep them rigidly together for insertion into the oxygen bottle. The taps and the capillary tubes can be held to the stand by simple clamps, if necessary. The

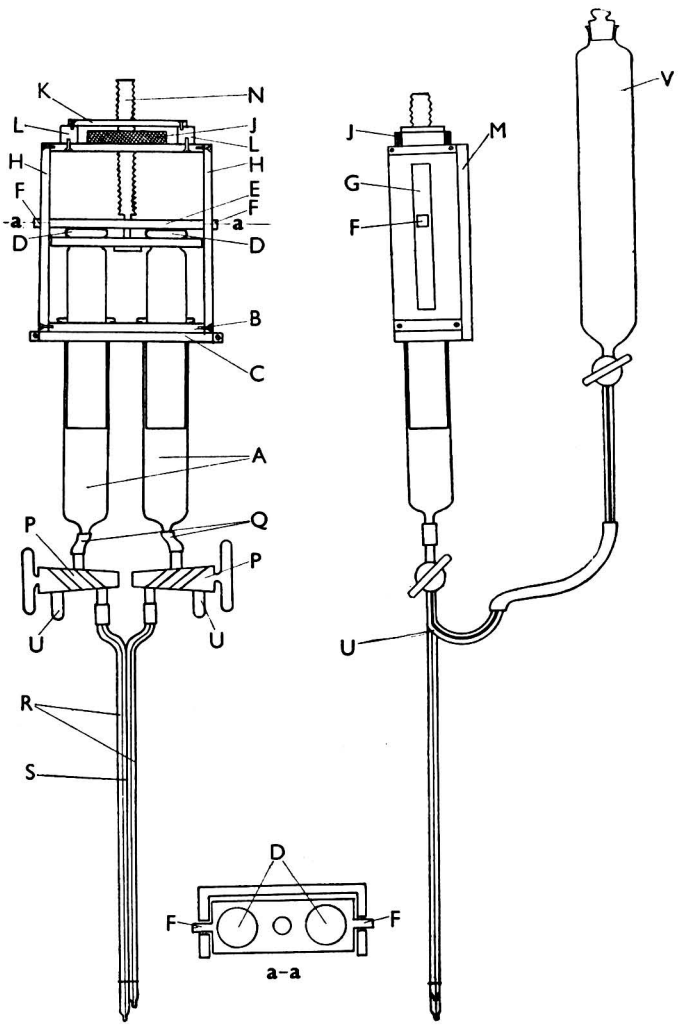


Fig. 1. The double-syringe pipette

two outside lower limbs, U, of the two-way cocks are bent back and connected by means of rubber tubing to two 50-ml separating funnels, V, mounted side by side in clamps on the stand above the level of the pipette.

OPERATION—

The two reagents are contained in the two reservoir funnels and are put in connection with the syringe pipette by appropriate adjustment of the taps with the plungers screwed down against the end of the syringes; the nut, J, is screwed up and the syringes are filled. The taps are then turned and the capillary tubes for delivering the reagents put in connection with the syringes; after

expulsion of any air bubbles the apparatus is ready for use. It is essential to have no extra pressure in the pipette and it is desirable to wait a few minutes after adjusting before adding the reagents. The two capillary delivery tubes are inserted quickly into the oxygen bottle and three turns of the knurled screw, J, ensures delivery of the required amount of each reagent. It is necessary to wash the ends of the delivery tubes between successive additions with water from a wash bottle. Twelve consecutive additions can be made in this way.

When not in use the syringes should be washed free of reagent either by repeatedly flushing with water from the reagent storage vessels or by partly dismantling the apparatus and manually washing them.

Calibration—

It is necessary to know the volume delivered for, say, three turns of the nut, so that the reagent strengths can be adjusted and the appropriate volume correction applied to the bottle; for this correction to be accurate successive turns of the nut must deliver the same volume. That the accuracy of successive deliveries was adequate for the purpose was shown by filling the pipette with distilled water and delivering several volumes by successive triple turns of the screw, the deliveries from the two sides being collected separately and weighed. One side delivered an average of 0·946 ml with a standard deviation of 0·0077 ml and the other side an average of 0·950 ml with a standard deviation of 0·0105 ml.

Application—

In the standard method for sea-water analysis, addition of 0·5 ml of a 40 per cent. manganous sulphate solution is followed by 1 ml of the potassium iodide - potassium hydroxide solution. Therefore a 20 per cent. solution of manganous sulphate was used in the pipette. In addition to making the addition of the reagents easier, the use of the syringe leads to greater accuracy, as additions are made much more rapidly and consequently with less exposure to air; increased accuracy has been found when dealing with waters of low oxygen content. The oxygen content of a set of replicate samples of sea-water, first, after standing in contact with air, and secondly, after de-oxygenation, were determined. The values are shown in Table I.

Table I

Oxygen content of samples of sea-water

	Mean oxygen content at S.T.P.	
	by standard technique, ml per litre	by new syringe pipette, ml per litre
Samples left in air	4·49 (s.d. 0·12)	4·46 (s.d. 0·08)
De-oxygenated samples	0·80 (s.d. 0·05)	0·75 (s.d. 0·03)

Under both conditions the oxygen content was lower by the proposed method, the difference being greater with the water of low oxygen content. By the standard method such errors would be expected to increase when the working conditions deteriorate. Further, for both examples the standard deviation is less with the proposed technique, which indicates less variability in the titration of the replicate samples.

The strengths of the reagents and the amounts added might need adjustment for other natural waters in order to obtain a satisfactory precipitate for oxygen absorption.

I wish to thank Mr. J. N. Thomson for his skilled assistance.

The Marine Station
Millport, Scotland

H. Barnes
December 12th, 1952

Ministry of Food

FOOD STANDARDS COMMITTEE

FLUORINE

THE Minister of Food has approved for publication a Report of the Food Standards Committee's Metallic Contamination Sub-Committee recommending that the following limits for the fluorine content of acidic phosphates used for food purposes and of foods containing acidic phosphates should replace those contained in the Fluorine in Food Order, 1947—

	ARTICLES OF FOOD	FLUORINE CONTENT
(*i*)	Acidic phosphate	30 p.p.m.
(*ii*)	Baking powder, including golden raising powder	10 p.p.m.
(*iii*)	Self-raising flour or any similar mixture (not included in item (*ii*) above) containing a farinaceous substance and an acidic phosphate	3 p.p.m.

The Sub-Committee points out that the limits placed on the fluorine content of these substances in 1947 were unavoidably high, as a part of the acid calcium phosphate used for food purposes at that time was manufactured from rock phosphate with a high natural fluorine content. This is no longer necessary, and the Sub-Committee considers that it should be possible with reasonable precautions to keep the fluorine content of acidic phosphate produced from elemental phosphorus within a limit of 30 p.p.m., and of baking powders within a limit of 10 p.p.m. The limit of 3 p.p.m. proposed for self-raising flour makes allowance for any fluorine that may be contributed by the flour or by creta and for the difficulty of ensuring a uniform distribution of creta in flour.

The Sub-Committee makes no recommendations with regard to the fluorine content of other foods.

The members of the Metallic Contamination Sub-Committee are: Mr. G. G. Barnes (Chairman), Professor S. J. Cowell, Dr. J. M. Johnston, Dr. W. P. Kennedy, Dr. G. W. Monier-Williams, Dr. J. R. Nicholls, Dr. G. Roche Lynch, Mr. G. Taylor and Mr. B. W. Smith (Secretary).

Any representations that interested parties may wish to make on the Sub-Committee's recommendations should be addressed to the Secretary of the Metallic Contamination Sub-Committee of the Food Standards Committee at the offices of the Ministry of Food, Food Standards and Hygiene Division, Great Westminster House, Horseferry Road, London, S.W.1.

The closing date for the recipt of representations is August 31st, 1953.

The Report is dated February, 1953.

ANTIOXIDANTS

THE Minister of Food has approved for publication a Report* of the Food Standards Committee's Preservatives Sub-Committee recommending that the Public Health (Preservatives, etc., in Food) Regulations should be amended so as to deal with and authorise the addition of selected antioxidants to certain foods.

Antioxidants, *i.e.*, substances that retard or prevent the development of oxidative rancidity, are permitted to be added to foods in some other countries, and it seemed desirable to establish the position of this class of substance under the Preservatives Regulations.

It is pointed out in the Report that, even under modern conditions of good manufacturing practice, development of rancidity in foods cannot always be avoided, particularly when fat-containing foods have to be stored for a long time.

The Sub-Committee has confined its attention to those antioxidants that are permitted in other countries, and has taken into account the biological, physiological and functional properties of such substances, and the needs of the food industry. In the light of these considerations the Sub-Committee has recommended that antioxidants should not be added to any foods other than to edible oils and fats (but not including butter) and to essential oils, to which it should be permissible to add either or both of the following antioxidants, in quantities not exceeding those stated—

Propyl gallate	0·01 per cent.
Butylated hydroxyanisole	0·02 per cent.

It is proposed that these recommendations should be subject to periodical review.

The Sub-Committee's recommendations do not preclude the use in foods of such substances as tocopherols, lecithin, and citric, tartaric and ascorbic acids, which are normal constituents of many foods, or of *d-iso*ascorbic acid, all of which have some antioxidant properties; nor do they preclude the packaging of foods in inert gases such as nitrogen or carbon dioxide.

The members of the Preservatives Sub-Committee are: Professor E. C. Dodds (Chairman), Mr. C. A. Adams, Mr. P. N. R. Butcher, Professor S. J. Cowell, Mr. A. Glover, Dr. E. B. Hughes, Dr. J. M. Johnston,

* The full text of the Report is published in the Ministry of Food Bulletin No. 706, dated June 13th, 1953, obtainable from H.M. Stationery Office, price 6d.

Dr. H. E. Magee, Dr. J. R. Nicholls and Dr. G. Roche Lynch. The Joint Secretaries are Mr. W. A. Godby and Mr. B. W. Smith.

Any representations that interested parties may wish to make on the Sub-Committee's recommendations should be addressed to the Joint Secretary of the Preservatives Sub-Committee of the Food Standards Committee at the offices of the Ministry of Food, Food Standards and Hygiene Division, Great Westminster House, Horseferry Road, London, S.W.1.

The closing date for the receipt of representations is September 30th, 1953.

The Report is dated May, 1953.

CURRENT STATUTORY INSTRUMENTS AND STATUTORY RULES AND ORDERS RELATING TO FOOD

The Index of Current Statutory Instruments and Statutory Rules and Orders, Sectional List No. 33, *has been revised to March* 31*st*, 1953, *and can be obtained from H.M. Stationery Office at cost of postage. See Analyst*, 1953, **78**, 257.

British Standards Institution

NEW SPECIFICATION*

B.S. 748:1953. Haemacytometer Counting Chambers and Dilution Pipettes. Price 3s. 6d.

AMENDMENT SLIP*

A printed slip bearing Amendments to a British Standard has been issued by the Institution, as follows—

PD 1612—Amendment No. 3 (May, 1953) to B.S. 541 : 1934. Determining the Rideal-Walker coefficient of disinfectants.

DRAFT SPECIFICATION

A FEW copies of the following draft specification, issued for comment only, are available to members of the Society, and can be obtained from the Secretary, Society of Public Analysts and Other Analytical Chemists, 7–8, Idol Lane, London, E.C.3.

Draft Specification prepared by Technical Committee FCC/4—Solvents and Allied Products.

CR(FCC)4068—Draft B.S. for the Karl Fischer Method for the Determination of Water.

CHANGE OF ADDRESS

THE British Standards Institution announce that from Monday, August 17th, 1953, their new address will be 2, Park Street, London, W.1 (Telephone: MAYfair 9000).

Book Reviews

INTRODUCTION TO THE STUDY OF PHYSICAL CHEMISTRY. By LOUIS P. HAMMETT. Pp. xii + 427. New York and London: McGraw-Hill Book Co. Inc. 1952. Price $6.00; 51s.

This is not an introductory textbook of physical chemistry. The author defines physical chemistry as a method of using quantitative and mathematical ways of thinking to attack the problems of chemistry, and a physical chemist as a scientist who is skilled and habituated in the use of quantitative and mathematical methods in approaching chemical problems. This book, then, is a guide—and a very good guide—to the application of mathematics to a wide range of experimental data in chemistry, a work dealing with what is generally spoken of as calculations in physical chemistry. As the author states, one can learn physical chemistry (as defined above) only by solving problems that involve the application of mathematics to quantitative data, and so a large number of problems form the most important part of the book. The student of physical chemistry will find it a most valuable exercise to work through the problems given here alongside of his textbook of physical chemistry, but the high price of the book will, it is to be feared, form a considerable barrier to its use in this country. ALEX. FINDLAY

STATISTICAL METHOD IN BIOLOGICAL ASSAY. By D. J. FINNEY, M.A., Sc.D. Pp. xix + 661. London: Charles Griffin & Co. Ltd. 1952. Price 68s.

This book sits appropriately on the shelf alongside Emmens's "Principles of Biological Assay." Even though the new volume contains three and a half times as many pages and words as the

* Obtainable from the British Standards Institution, Sales Department, 2, Park Street, London, W.1.

former, and takes proportionately more room, they must be regarded as complementary and in a sense convergent. Emmens is a biologist who had to face quantitative problems and therefore found it essential to acquire statistical techniques: Finney is a statistician whose advice has so often been sought by biologists (including bio-assayists) that he has been compelled to study, and has as a result clearly become much interested in, their particular problems.

In spite of their differences in approach, and the disparity in size, these books should both be in the libraries of all involved in biological assay or quantitative pharmacology. There is even more mathematics in the new book than in the earlier one, but it is by no means free of those slightly epigrammatic admonitions that we have come to expect in the written or spoken word of Dr. Finney. For example, in his chapter (15) on "Validity and the Choice of Metameter" he discusses at a little length the level of probability at which data (I think he means *results* obtained by an experimenter, not the figures given to him) may be regarded as "suspicious" and therefore be rejected, and writes, "on the basis of his past experience . . . he must decide whether or not he believes the assay to be valid. Statistical analysis cannot enable him to evade his responsibility, but can ensure that all relevant evidence is presented in a clear, objective manner." This sentence is, incidentally, a fair example of the author's clean and logical style. Apart from his use of certain terms (an example is indicated above), the most pedantic critic can find little fault with his writing.

But something must be done about the word "sensitivity." Dr. Finney writes (p. 92) about the "sensitivity of the subjects to variations in dose" and later in the same paragraph that "The regression coefficient measures the sensitivity of subjects to changes in dose." Apart from the incidental point that he surely means "represents," not "measures," these sentences (and others) involve giving to the word a meaning entirely different from that with which it is used by pharmacologists, to say nothing of chemists and physicists. To all of these the concept is associated with that of a threshold effect, or at any rate with that of the extent of a response to a given stimulus, as for example the median effective dose, when quantal responses are involved, and not, as Dr. Finney would have it, to the concept of the increase in response to a given increase in dose. How this confusion arose—for this I am sure it is, and it may alas lead to confusion worse confounded—is a nice point for those interested in methodology and semantics. But this is hardly the place to discuss it.

Meanwhile let it be said clearly that Dr. Finney's book, which has been long and eagerly awaited, is certainly the "comprehensive account of designs and statistical analyses" claimed by the publisher. Those who wish to extract information from quantitative work with living plants or animals, or "surviving" tissues of such organisms, that is, with any material having an appreciable tendency to biological variation, simply must have this book as a reference guide, as a mathematical philosopher and as an expository, and sometimes gently expostulatory, friend. It will not solve all the bio-assayist's problems. Why should it? But it will give him a pretty shrewd idea of whether any particular problem can be solved and, if so, what will be the most efficient means of those available.

A. L. BACHARACH

Publications Received

FERTILIZER EXPERIMENTS, 1927–46, AT THE KROONSTAD AGRICULTURAL RESEARCH STATION. Science Bulletin No. 319, Chemistry Series No. 194. By J. VAN GARDEREN, D.Sc., A.R.I.C., and I. J. SMUTS, B.Sc. Pp. 55. Pretoria, South Africa: Department of Agriculture. 1952. Price 6d.

AGRONOMY EXPERIMENTS, 1927–46, AT THE KROONSTAD AGRICULTURAL RESEARCH STATION. Science Bulletin No. 320, Agricultural Education and Research Series No. 12. By I. J. SMUTS, B.Sc., and J. VAN GARDEREN, D.Sc., A.R.I.C. Pp. vi + 44. Pretoria, South Africa: Department of Agriculture. 1952. Price 6d.

AGRONOMY EXPERIMENTS, 1938–45, AT THE VAAL-HARTZ AGRICULTURAL RESEARCH STATION. Science Bulletin No. 321, Education and Research Series No. 13. By W. VAN DER MERWE, B.Sc., and J. VAN GARDEREN, D.Sc., A.R.I.C. Pp. 71. Pretoria, South Africa: Department Agriculture. 1952. Price 6d.

FERTILIZER EXPERIMENTS IN NATAL, 1933–50. Science Bulletin No. 336, Chemistry Series No. 196. By J. VAN GARDEREN, D.Sc., A.R.I.C., J. G. BREVIS, B.Sc., and A. J. TAYLOR, M.Sc., A.R.I.C. Pp. ii + 38. Pretoria, South Africa: Department of Agriculture. 1952. Price 6d.

Notice to Authors

THE Society publishes papers concerned with all aspects of analytical chemistry, inorganic and organic, including chemical, physical, biological, bacteriological and micro-methods. Papers on these and allied subjects, by members of the Society or by non-members, may be submitted for publication; they may—

(1) record proposals for new methods and the investigations on which the proposals are based;

(2) record the results of original investigations into known methods or improvements therein;

(3) record analytical results obtained by known methods where these results, by virtue of special circumstances, such as their range or the novelty of the materials examined, make a useful addition to analytical information;

(4) record the application of new apparatus and new devices in analytical technique and the interpretation of results;

(5) record minor investigations or kindred matter and descriptions of new apparatus and its applications, which may be accepted for publication under their respective section headings.

Communications—Papers and all communications relating thereto should be sent to the Editor of *The Analyst*, 7–8, Idol Lane, London, E.C.3.

Papers will normally be submitted to at least one referee, by whose advice the Publication Committee will be guided as to the acceptance or rejection of any communication.

Papers accepted by the Publication Committee must not be published elsewhere except by permission of the Committee.

Synopsis—All papers must be accompanied by a short synopsis of about 100 to 250 words indicating the scope and results of the investigation, and an appraisal of the accuracy of any new method proposed. Contributions intended for publication in the "Notes" or "Apparatus" sections do not require synopses.

Proofs—The address to which proofs are to be sent should accompany the paper. Proofs should be carefully checked and returned within 48 hours.

Reprints—Twenty-five reprints, or a maximum of fifty if there is more than one author, are supplied gratis. Additional reprints may be obtained at cost if ordered directly from the printers, W. Heffer & Sons Ltd., Hills Road, Cambridge, at the time of publication. Details are sent to authors with the proofs.

NOTES ON THE WRITING OF PAPERS FOR "THE ANALYST"

Manuscript—Papers and Notes should be typewritten with double spacing and on one side *only* on non-absorbent paper. Two copies should be sent to the Editor, and a further copy retained by the author. A duplicate sketch or tracing of all figures should be included for office use. Manuscripts should be in accordance with the style and usages shown in recent copies of *The Analyst*.

The title should be brief but descriptive.

Conciseness of expression should be aimed at; clarity is facilitated by the adoption of a logical order of presentation, with the insertion of suitable paragraph or sectional headings.

Descriptions of new methods should be supported by experimental data showing their precision and selectivity.

Generally, the best order of presentation is as indicated below—

(*a*) Synopsis.

(*b*) Statement of object of investigation and, if necessary, historical introduction.

(*c*) Preliminary experimental work.

(*d*) Description of method. Working details of proposed methods are most concisely and clearly given in the imperative mood, and should normally be given in this form, *e.g.*, "Dissolve 1 g of sample in 10 ml of water and add . . ." Well-known procedures must not be described in detail.

(*e*) Presentation of results.

(*f*) Scope and validity of results.

(*g*) Conclusions, and if required, a short summary of the principal results.

Tables, diagrams, etc.—The number of tables should be kept to a minimum. Column headings should be brief. Tables consisting of only two columns may often be arranged horizontally. No lines should be ruled in tables in the manuscript. Tables should be supplied with titles and be so set out as to be understandable without reference to the text.

Tables or graphs may be used, but not both for the same set of results, unless important additional information is given by so doing.

In general, graphs should have a reasonable number of co-ordinate lines, and not only the two main axes. Graphs consisting of straight lines passing through the origin, such as calibration curves, should not be submitted: instead, an equation should be given in the text. Graphs should be prepared in the same manner as other diagrams.

Diagrams and graphs should be drawn in Indian ink on Bristol board, stout paper or tracing cloth, not larger than foolscap size. The use of squared paper should be avoided if possible, as it often leads to poor reproduction. Red, orange or brown ruled paper must not be used; if it is necessary to use ruled paper, blue or green rulings may be used, as these colours do not reproduce in block-making. All lettering should be inserted lightly in black lead pencil at the appropriate place on the diagram, and will be replaced by type in block-making. All lines in Indian ink should be firmly drawn and sufficiently thick to stand reduction. If the artist is uncertain in his free-hand work on graphs, he should submit a careful pencil drawing that can be traced.

Drawings should be specially prepared for submission to *The Analyst*, as they cannot normally be returned, and may be modified or cut in the course of block-making.

Photographs—Photographs for reproduction on art paper should be submitted in the form of glossy prints made to give the maximum detail.

Abbreviations—Normality and molarity are generally expressed as decimal fractions (*e.g.*, 0·02 *N*, 0·375 *M*). Abbreviational full stops are omitted after the common contractions of metric units (*e.g.*, ml, g, μg, mm) and after ° C, ° F, μ, A and other units represented by symbols; litre and metre, when without prefixes, are printed in full.

Abbreviations of words other than recognised units should be avoided in the text, symbols and formulae are not used instead of the names of elements and compounds in the text, but may be used in addition to names where it is necessary to avoid ambiguity, *e.g.*, to specify crystalline composition, as in $CuSO_4.5H_2O$, to show structure, or in equations.

Percentage concentrations of solutions should be stated as "per cent. w/w" (alternatively "g per 100 g"), as "per cent. w/v" (alternatively "g per 100 ml"), or as per cent. v/v. Concentrations of solutions of the common acids, however, are often conveniently given as dilutions of the concentrated acids; they should then be given in the form "diluted hydrochloric acid (1 + 4)," which signifies 1 volume of the concentrated acid mixed with 4 volumes of water. This avoids the ambiguity of 1:4, which might be equivalent to *either* 1 + 4 *or* 1 + 3.

References—References should be numbered serially in the text by means of superscript figures, *e.g.*, Dunn and Bloxam[1] or Allen,[2] and collected in numerical order under "REFERENCES" at the end of the paper. They should be given, with the authors' initials, in the following form—

1. Dunn, J. T., and Bloxam, H. C. L., *J. Soc. Chem. Ind.*, 1933, **52**, 189T.

2. Allen, A. H., "Commercial Organic Analysis," Churchill, London, 1882, p. 123.

For books, the publisher, and place and date of publication should be given, followed by volume or page number, or both if required.

Authors are recommended, in their own interests, to check their lists of references against the original papers; second-hand references are a frequent source of error.

Neglect of any of the points mentioned above is likely to cause delay in publication.

Further useful advice to authors is contained in the Royal Society's publication entitled "General Notes on the Preparation of Scientific Papers," published for the Royal Society by the Cambridge University Press, price 2s. 6d.

ANALYTICAL CHEMIST, B.Sc., required by London firm of Analysts. Applicants must have had varied experience of organic and inorganic commercial analysis. Write stating age, with full particulars and salary required, Box No. 3846, THE ANALYST, 47, Gresham Street, London, E.C.2.

THE DIRECTORS OF THE CO-OPERATIVE WHOLESALE SOCIETY LTD. invite applications for the position of Chief Chemist for their Preserves Group of Factories. Candidates must have experience in the trade, and the appointee would be expected to collaborate with management on raw material purchases and control, supervise routine laboratory work, and be capable of initiating and developing new lines in the various production departments involving Jams, Pickles, Sauces and Canning. Applications, stating age, education, experience, qualifications and salary required, should be addressed to the C.W.S. Ltd., Central Labour Department, 1, Balloon Street, Manchester, 4, endorsed "Chief Chemist—Preserve Factories."

EAST MALLING RESEARCH STATION

CHEMIST required for mineral analysis of plants. The work entails participation in routine analysis and supervision of assistants. Applicants for the post, which may be in the Assistant Experimental Officer or Experimental Officer grade, according to age and experience, should have a Higher National Certificate in Chemistry or equivalent qualification. Good experience of modern methods of analysis essential. Superannuation under the Ministry of Agriculture Scheme. Further particulars and application form may be obtained from the Secretary, East Malling Research Station, near Maidstone, Kent, by whom applications should be received not later than August 31st, 1953.

A MICRO-ANALYST is required for the Chemical Research Organisation to carry out quantitative organic microanalysis and to assist in the introduction of new and improved methods. Applicants should be aged about 25, possess a B.Sc. or A.R.I.C. qualification and preferably have experience in micro-analysis. Salary according to age and experience. Apply initially in writing, giving full details of qualifications and experience, to Personnel Officer, May & Baker Ltd., Dagenham, Essex, quoting reference No. 369.

HER MAJESTY'S COLONIAL SERVICE

VACANCIES exist for a Government Chemist and Assistant Government Chemist in the Colonial Chemical Departments of (*a*) Uganda (CDE. 97/9/01) and (*b*) Nigeria (CDE. 97/14/01).

QUALIFICATIONS:

(*a*) Fellowship of the R.I.C. (Branch E) and B.Sc. (Chemistry) 1st or 2nd class honours; three years' experience of analytical work in applied chemistry desirable.

(*b*) Fellowship or Associate of the R.I.C. (Branch E) or honours degree in Chemistry of British University or its equivalent.

DUTIES:

(*a*) (Chemist.) Routine chemical estimations and investigations; teaching of African staff; the care and accounting for Government stores and giving expert evidence in court.

(*b*) (Assistant Chemist.) General analytical, including bacteriological and chemical analysis of water and forensic work.

TERMS OF APPOINTMENT:

(*a*) On agreement for period of 30–36 months on pensionable terms with salary in the scale of £865–£1320 per annum, plus cost of living allowance of 30 per cent. of substantive salary subject to maximum of £300.

(*b*) Pensionable with salary in the scale of £570–£1290 per annum, plus overseas allowance of between £180 and £270 per annum.

Applications should be made in writing to the Director of Recruitment (Colonial Service), Colonial Office, Sanctuary Buildings, Great Smith Street, Westminster, S.W.1 (from whom further particulars can be obtained), quoting appropriate reference number and brief particulars of age, qualifications and experience.

CHELSEA POLYTECHNIC

MANRESA ROAD, LONDON, S.W.3

DEPARTMENT OF CHEMISTRY

A Course of Lectures and Practical Work on

THE CHEMISTRY AND MICROSCOPY OF FOOD, DRUGS AND WATER

will be given

SEPTEMBER, 1953 to JULY, 1954

Lecturer in Charge:

R. G. MINOR, Ph.C., F.R.I.C.

The course is based on the Syllabus of the Examination for the Fellowship of the Royal Institute of Chemistry (Branch E), and includes Analysis and Microscopy of Food, Drugs and Water, Toxicological Analysis, Acts and Regulations relating to Food, Drugs and Poisons.

Mr. Minor will attend on Wednesday, 23rd September, 1953, 6–8 p.m., to advise students.

Full particulars can be obtained on application to the Secretary.

Volume 78. No. 929, Pages 449-508 August, 1953

THE ANALYST

THE JOURNAL OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

CONTENTS

Editorial *Page*

Drummond Memorial Fund .. 449

Proceedings of the Society of Public Analysts and Other Analytical Chemists

The Faraday Society .. 450

The Bernard Dyer Memorial Medal .. 450

Original Papers

The Efficient Planning of Microbiological Assays—Eric C. Wood .. 451

The Selection of Methods for Routine Assays for Members of the Vitamin-B Complex—H. Pritchard .. 460

Some Observations on the Determination of Benzoyl Peroxide in Flour and Bread—R. A. Knight and D. W. Kent-Jones .. 467

A Rapid Method for the Determination of Molybdenum in Soils—J. L. Grigg .. 470

The Determination of Keten and Acetic Anhydride in the Atmosphere—W. M. Diggle and J. C. Gage .. 473

The Polarographic Determination of Iron and Chromium—M. Perkins and G. F. Reynolds 480

The Potentiometric Determination of Quadrivalent Tellurium by Potassium Permanganate in Weakly Alkaline Solutions—I. M. Issa and S. A. Awad .. 487

A Bromometric Method for the Determination of Formic Acid and Nitrite—Miss J. V. L. Longstaff and K. Singer .. 491

Notes

The Colorimetric Determination of Small Amounts of Fluorine—H. F. Liddell .. 494

The *p*-Hydroxydiphenyl Test for Acetaldehyde: The Determination of Alanine and Threonine in Protein Hydrolysates—B. F. Folkes .. 496

The Characterisation of Vinegar by its Albuminoid Ammonia Value—S. N. Mitra .. 499

The Decomposition of Ammonia in Sealed-Tube Micro-Kjeldahl Digestions with a Selenium Catalyst—P. R. W. Baker .. 500

Apparatus

A Double Syringe-Pipette for Dissolved Oxygen Estimations—H. Barnes .. 501

Ministry of Food .. 504

British Standards Institution .. 505

Book Reviews

Introduction to the Study of Physical Chemistry. By Louis P. Hammett .. 505

Statistical Method in Biological Assay. By D. J. Finney .. 505

Publications Received .. 506

Notice to Authors .. 507

Printed and Published for the Society of Public Analysts and Other Analytical Chemists by W. Heffer & Sons Ltd., Cambridge, England. **Communications to be addressed to the Editor, F. L. Okell, 7-8, Idol Lane, London, E.C.3.** Enquiries about advertisements should be addressed to Walter Judd Ltd., 47, Gresham Street, London, E.C.2.

Entered as Second Class at New York, U.S.A., Post Office.