THE ANALYST

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

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7-8, IDOL LANE, LONDON, E.C.3
Telephone: MANsion House 6608

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

Volume 76 Price 6s. 6d.
Subscription Rate, inclusive of Abstracts C, 80/- per annum, Post Free
No. 905, Pages 445-502 August, 1951

THE ANALYST

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

DEATHS

We regret to record the deaths of

Arthur James Curtin Cosbie
William Edmund Francis Powney.

PHYSICAL METHODS GROUP

The Thirty-second Ordinary Meeting of the Group was held at 4 p.m. on Tuesday, May 22nd, 1951, in the Lecture Room of the Institute of Physics, 47, Belgrave Square, London, S.W.1. Mr. B. S. Cooper was in the chair and forty-five members and visitors were present.

The following papers on Radiochemistry were presented and discussed: "Radio-active Tracer - Paper Chromatography Techniques," by F. P. W. Winteringham, F.R.I.C., A. Harrison and R. G. Bridges; "Paper Chromatography of Radio-active Penicillin," by E. Lester Smith, D.Sc., F.R.I.C., and D. Allison; "The Determination of Submicrogram Quantities of Arsenic by Radio-activation," by A. A. Smales, B.Sc., A.R.I.C., and B. D. Pate, B.Sc.; "Micro-determination of Sodium and Potassium by Activation Analysis," by R. D. Keynes, M.A.

The Precision of the 3-Point Correction Method of Spectrophotometric Assay of Vitamin A

By D. C. M. ADAMSON, W. F. ELVIDGE, N. T. GRIDGEMAN, E. H. HOPKINS, R. E. STUCKEY and R. J. TAYLOR

A seven-laboratory determination of $E^{1\%}_{1cm}$ at 328 mμ, geometrically corrected for absorption irrelevant to vitamin A, was carried out on each of five oils containing vitamin A. Readings were made in duplicate on photo-electric instruments. From a statistical analysis of the results it is concluded that the limits of error of a determination in duplicate from any one laboratory are about ± 15 per cent. for $P = 0{\cdot}05$. The corresponding figure for gross, *i.e.*, uncorrected, E values is about ± 2 per cent.

The practice of taking the vitamin-A content of any oil to be directly proportional to its absorption of ultra-violet light at 328 mμ is moribund, despite the fact that within certain classes of oils and concentrates an empirical relation between the two can be assumed with some degree of confidence and roughly checked by colorimetric reactions; for an analytical method, the interpretation of which depends on extraneous information, does not lend itself to standardisation and so must eventually be laid aside in favour of some more nearly absolute method—even if the new method is technically more complex. Most new methods now being developed, and some already in use, for the estimation of vitamin A depend on the elimination of, or allowance for, the ultra-violet absorption due to the presence in the sample of compounds other than the vitamin. Knowing the spectrophotometric characteristics of the

pure vitamin in the particular solvent used, we can then calculate the vitamin-A content of the sample by simple proportion. In practice it is customary to express the result not in percentages (parts per 100) but in International Units per gram (parts per $10^7/3$), the I.U. being 0·3 μg of vitamin A as alcohol.[1]

The ingenuity and simplicity of one method of allowing for irrelevant absorption have earned it considerable attention. This is the Morton - Stubbs 3-point geometric correction method.[2,3,4,5] Its basic assumption is that the irrelevant absorption of most oils and concentrates is linear at three points in the regions 310 to 313 mμ, 325 to 328 mμ and 336 to 339 mμ. Now it conveniently happens that there are points within the same regions on the absorption curve of vitamin A itself whose ordinates bear the relation 6:7:6 and this makes for a simple geometry. The exact points chosen and the constants of the correction formula depend upon whether the whole oil or the unsaponifiable fraction is being examined and upon the solvent used. For the whole oil in *cyclo*hexane the formula is—

$$\text{E at } 328\ m\mu \text{ (corr.)} = 7 \times \text{E at } 328\ m\mu - 2{\cdot}882 \times \text{E at } 313\ m\mu - 4{\cdot}118 \times \text{E at } 338{\cdot}5\ m\mu.$$

Hence the substitution of the Morton - Stubbs method for the old method involves only the taking of two extra spectrophotometric readings on the same solution and a consequent calculation.

The theory of the correction procedure is discussed elsewhere[6]; the present paper is concerned with its reproducibility, a matter that does not yet seem to have been investigated.

Experimental

Seven independent laboratories undertook to determine, by photo-electric spectrophotometry, $E^{1\%}_{1cm}$ at 313 mμ, 328 mμ and 338·5 mμ, each in duplicate (separate weighings), on five oils, the solvent to be *cyclo*hexane. As a subsidiary investigation, they were asked for complete ultra-violet absorption curves between about 250 mμ and 350 mμ. This secondary information was mainly for another purpose,[6] but in the present context it served to show that none of the five curves conformed to the requirement[1] that it should "agree closely with that of the international standard measured under the same conditions and compensated with a solution of the diluent oil," and in particular that intensities of absorption "in the region 310 to 350 mμ expressed as decimal factions of the maximum should not differ between sample and standard by more than 0·02." As these requirements were not met, the curves were presumed to contain irrelevant absorption and to be in need of rectification. Not all the absorption curves rose to a maximum at exactly 328 mμ, but none of the maxima fell outside the critical range 325 to 328 mμ. For the sake of simplicity the figure 328 will be used in the remainder of this paper for all the maxima.

Four of the samples were—

A refined fish-liver oil concentrate, E at 328 mμ (gross) ..	=	154·4
A halibut-liver oil, E at 328 mμ (gross)	=	16·61
A hake-liver oil, E at 328 mμ (gross)	=	6·31
A cod-liver oil, E at 328 mμ (gross)	=	0·628

The fifth sample was a blend of these oils. Its composition was afterwards revealed as 20 parts of concentrate, 31 parts of halibut-liver oil, 20 parts of hake-liver oil and 30 parts of cod-liver oil. Its gross E at 328 mμ could therefore be expected to be 37·11; the average value found was reasonably close, *viz.*, 36·82.

Results

Before application of the correction formula, the submitted estimates of the gross $E^{1\%}_{1cm}$ at 328 mμ for the five samples were themselves statistically analysed, and a coefficient of variation of 1·48 for any one estimate in one laboratory emerged. Additionally, the corresponding coefficient for estimates at any of the three wavelengths was extracted and found to be 1·26. These are normal values and compare favourably, for instance, with the figure of 1·55 found in a recent investigation of the basic limits of error of photo-electric instruments.[7]

The correction formula was then applied to each triad of E estimates. The mean results in the form of percentage variation per sample are shown in Table I.

The values in Table I range from 92·3 to 117·2. As expected, the individual estimates (not tabulated) show a wider range. Not all the single estimates were available; three of the seven laboratories submitted averages of duplicate estimates only, and this necessitated a

correspondingly restricted estimate of the residual coefficient of variation. The essentials of the analysis of variance of the corrected E values are set out in Table II.

TABLE I

ESTIMATES OF CORRECTED $E^{1\%}_{1\,cm}$ AT 328 mμ ARRANGED AS PERCENTAGES OF OVER-ALL AVERAGES, EACH ENTRY BEING THE MEAN OF TWO ESTIMATES

Laboratory	Concentrate, %	Halibut-liver oil, %	Hake-liver oil, %	Cod-liver oil, %	Blend, %
A	93·8	117·2	94·9	97·5	107·2
B	104·8	92·3	104·6	104·9	111·7
C	100·5	99·0	100·4	105·9	93·5
D	100·9	98·5	100·5	104·8	103·8
E	95·7	96·7	97·1	97·1	93·7
F	102·6	95·9	97·6	92·6	95·4
G	101·6	100·4	104·9	97·2	94·7
Means	100	100	100	100	100
Absolute mean E values	138·7	15·21	5·62	0·541	34·80

TABLE II

ANALYSIS OF VARIANCE OF THE ESTIMATES OF CORRECTED $E^{1\%}_{1\,cm}$ AT 328 mμ

Source of Variance	D.F.	Mean square
Between laboratories	6	77·49
Laboratories × oils	24	68·61
Residual error	20	161·54

Although the interaction term "laboratories × oils" in Table II has a mean square lower than that for the residual error, it will be wise to take it into account inasmuch as it covers more of the experimental data than does the residual term. We may therefore combine the two estimates and hence take the square root of the weighted mean square of 68·61 and 161·54, *i.e.*, 10·5, as the fairest estimate of the residual coefficient of variation.

The results of the trial may be presented in another form as the percentage of irrelevant absorption at 328 mμ estimated by correction. Table III shows these means. The expected values for the blend are calculated from each laboratory's findings on the four main oils and the known composition of the blend. The seven laboratories are entered in descending order of agreement between the values found and expected for the blend.

TABLE III

ESTIMATES OF PERCENTAGE OF IRRELEVANT ABSORPTION AT 328 mμ BY THE CORRECTION METHOD

Each figure represents the mean of at least two estimates

Laboratory	Concentrate, %	Halibut-liver oil, %	Hake-liver oil, %	Cod-liver oil, %	Blend	
					Found, %	Expected, %
G	6·9	9·6	5·4	16·0	7·1	7·0
C	10·3	13·8	12·1	11·6	12·2	10·9
F	3·6	13·3	10·9	16·7	6·8	5·2
D	10·9	9·7	7·7	11·8	7·5	10·6
E	11·8	13·2	12·6	13·9	8·1	12·0
A	17·0	−4·8	17·0	16·3	7·0	14·0
B	10·7	20·3	10·3	13·1	4·0	12·0
Means	10·2	10·7	10·9	14·2	7·5	10·2

The distribution of results in Table III and, in particular, the results for the blend give further credence to the derived errors. It is interesting to note that analysis of variance of the contents of Table III shows no significant difference between samples; if all the oils had in truth contained the same amount of irrelevant absorption (on the "correction" criteria) a distribution such as that now found would occur in 1 in 5 trials of this size. It might strictly be claimed that, as far as these oils are concerned, the gross E value provides

a measure of the presumptive relative potency that is as good as the corrected value. In fact, however, the tendency for the cod-liver oil figure to be higher, and perhaps to be more consistent, cannot be ignored, especially as there is some evidence[6] to show a fair reliability of the method when applied to this type of oil.

Discussion of results

That this estimate of 10·5 for the residual coefficient of variation of one result in one laboratory is not unreasonable can be inferred from a theoretical consideration of the influence of observational errors on corrected E values.[6] The magnification predicted is of the order of 9·5, so that from the coefficient of variation found for the gross estimates (see above), 1·25, we should expect the coefficient for the corrected values to be nearly 12.

It is important to make clear the meaning of this coefficient. It concerns the variations in the *relative* readings of the three optical densities from one test solution to another. Inter-laboratory discrepancies in the calibration of instruments and experimental errors in the preparation of test solutions are not directly concerned; these are factors that operate equally at all three wavelengths, and the calculation of the corrected result involves no magnification of this type of error.

In this particular set of results no significant inter-laboratory differences arose (see Tables I and II). This is unusual; work by the Photo-electric Spectrometry Group, published in part,[7] indicates that, in general, inter-laboratory (more strictly, perhaps, inter-instrument) differences can be represented by a coefficient of variation of about 1·6. If we take the published value of 1·62 and the companion value of 1·55 for the residual coefficient of variation of one result on one test solution, it follows that the standard deviation of any one duplicated assay in one laboratory is—

$$\sqrt{\left(1{\cdot}62^2 + \frac{1{\cdot}55^2}{2}\right)},\ i.e.,\ \pm 2{\cdot}0 \text{ per cent.},$$

which means (*a*) that two-thirds of all such duplicates, each from a different laboratory, will fall within the range 98 to 104 per cent. of the over-all means, and (*b*) that there is a probability of 2 in 3 that the true result, defined as the mean assay from an infinite number of laboratories, lies within the range 98 to 104 per cent. about any one (duplicate) result. On the same basis, the corresponding deviation for corrected E values can be expected to approximate to—

$$\sqrt{\left(1{\cdot}62^2 + \frac{(1{\cdot}55 \times 9{\cdot}5)^2}{2}\right)},\ i.e.,\ \pm 10{\cdot}5 \text{ per cent.}$$

The value found for this deviation of duplicates in the present investigation (with no significant inter-laboratory differences) is ±7·4 per cent., and this is probably a fair guide (erring, if at all, on the low side) to what can normally be expected. The equivalent P = 0·05 limits of error are ±15 per cent. (again for the mean of two estimates from one laboratory).

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January, 1951

Observations on the Spectrophotometric Assay of Vitamin A by Geometric Correction of Absorption Curves

By N. T. GRIDGEMAN

The accuracy of the spectrophotometric assay of vitamin A by geometric correction of absorption curves depends on the valid scope of two assumptions, *viz.*, that the absorption curves of natural forms of the vitamin are indistinguishable from that of pure all-*trans* vitamin A, and that the ultra-violet absorption curves of materials other than vitamin A in natural oils have three linear points at certain wavelengths. There is evidence that these assumptions are not always correct, and it is shown that comparatively small departures from these conditions may be associated with appreciable loss of accuracy. An attempt is made to quantify degrees of departure in terms of the accuracy of the result. The influence of the normal observational errors of spectrophotometry on the precision of the result is also considered. The application of the method to cod-liver oil, as originally proposed, appears to be more firmly based than its application to richer oils.

In 1946, Morton and Stubbs[1] described a geometric method of "breaking down" compound spectrophotometric absorption curves into two parts, the major part being the established and characteristic curve of the compound in the original mixture whose quantitative analysis

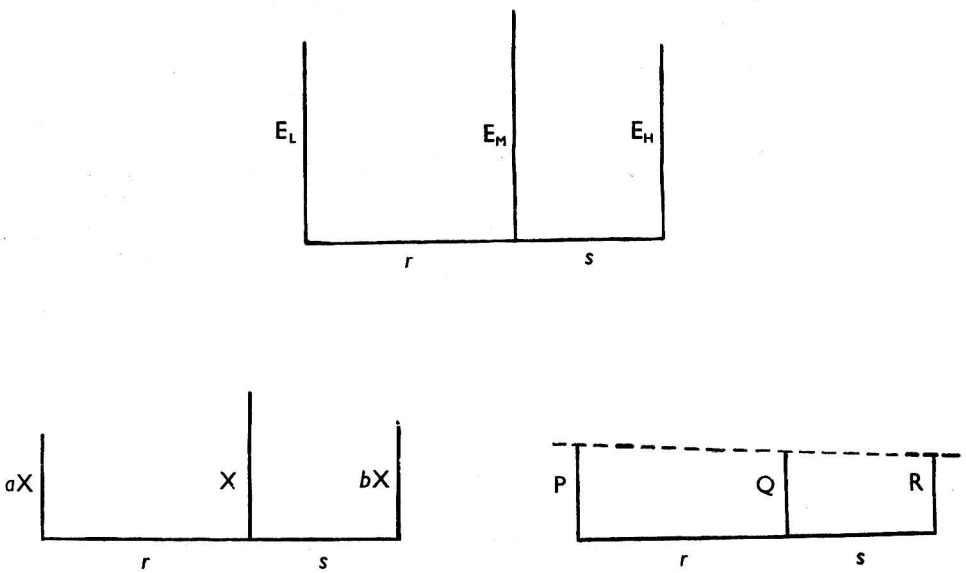

Fig. 1. The geometry of correction

is sought and the minor part being the remaining "irrelevant" absorption about whose shape certain simple assumptions must be made. The method was applied in particular to the estimation of vitamin A in cod-liver oil. Later, the same authors extended their work to the analysis of other liver oils.[2,3] Oser[4] and McGillivray[5] have also published papers on the application of the method to oils containing vitamin A. The present paper records some observations on the theory and on the reliability of the method as applied to the analysis of vitamin A in fish-liver oils and concentrates; in this context, reliability includes both precision (reproducibility of results) and accuracy (approximation to truth).

Derivation of method

Consider three vertical parallel lines, as shown in Fig. 1, of length E_L, E_M and E_H, rising from a horizontal base at distances apart r and s. Suppose these verticals to be the sum of the two lower sets, about which we know, first, the values a and b in set I, and secondly, that the tops of the three verticals in set II fall on a straight line. Given E_L, E_M and E_H, r, s, a and b, we are required to find X. It can easily be shown that—

$$X = \frac{(r+s)\left[E_M - E_L\frac{s}{r+s} - E_H\frac{r}{r+s}\right]}{r(1-b)+s(1-a)}, \quad \ldots \quad (1)$$

and the expressions for aX, bX, P and Q and R readily follow.

Applying this to spectrophotometry we can see that, given (*i*) the optical densities (verticals) at any three wavelengths (horizontals) of a solution of a mixture, (*ii*) the relative optical densities at those wavelengths of one component (α) of the mixture and (*iii*) the fact that the optical densities at the same wavelengths of the other component, or the sum of the other components (β), lie on a straight line, we can calculate for each wavelength the exact and unique partition of the original optical density between the contributions of α and β. Further, knowing the cell thickness, the strength of the solution and the light absorption by pure α at the three wavelengths, we can transpose our results into quantitative analysis. In practice this means working in terms of $E^{1\%}_{1cm}$. It is important to note that there is no assumption of linearity of the optical densities of the irrelevant component β at wavelengths other than the specified triad; in between them the optical densities may take any value.

In practice we can simplify things by so choosing r and s in equation (1) that $a = b =$ (say) k. Then—

$$X = \frac{1}{1-k}\left[E_M - E_L\frac{s}{r+s} - E_H\frac{r}{r+s}\right] \quad \ldots \quad (2)$$

To apply this equation to the assay of vitamin A, Morton and Stubbs determined the relative optical densities in various solvents over a wide wavelength range for vitamin-A_1 acetate (assumed to have precisely the same curve as the fatty-acid esters of vitamin A existing in fish-liver oils) and for vitamin-A_1 alcohol (assumed to have the same curve as the free vitamin A that is split off into the unsaponifiable matter of fish-liver oils). They then assumed that, in most fish-liver oils, "the irrelevant absorption 'curve' is linear over the approximate range 310 to 340 mμ, *i.e.*, that no impurity or artefact shows a maximum very close to that of vitamin A," adding that "a good deal is now known concerning the spectra of vitamin A_2 and oxidation products of vitamin A, and it can be said that over the range 310 to 340 mμ their absorption curves are at least approximately linear."[3] With this in mind they chose, for vitamin-A acetate in *cyclo*hexane, $r = 15$ and $s = 10{\cdot}5$ on either side of $\lambda_{max.} = 328$ mμ. So in the present notation, $E_M = E$ at 328 mμ, $E_L = E$ at 313 mμ, and $E_H = E$ at 338·5 mμ, where E is an abreviation for $E^{1\%}_{1cm}$. These fixation points give $k = 6/7$, and the equation becomes—

$$X = 7\left[E_M - \frac{7}{17}E_L - \frac{10}{17}E_H\right]$$

$$= 7[\text{E at } 328\,m\mu - 0{\cdot}4118 \text{ E at } 313\,m\mu - 0{\cdot}5882 \text{ E at } 338{\cdot}5\,m\mu], \quad \ldots \quad (3)$$

X, of course, being E at 328 mμ (*net*), or (*corr.*), *i.e.*, "corrected" to eliminate all absorption other than that due to vitamin A, in contradistinction to the E at 328 mμ in the brackets, which is gross, *i.e.*, as recorded on the original sample.

Other, very similar, equations hold for other conditions. For vitamin-A alcohol in *cyclo*hexane, Morton and Stubbs find—

$$X = 7\left[E_M - \frac{2}{5}E_L - \frac{3}{5}E_H\right]$$

$$= 7[\text{E at } 326\,m\mu - 0{\cdot}4 \text{ E at } 311\,m\mu - 0{\cdot}6 \text{ E at } 336\,m\mu].$$

Oser[4] finds that the above Morton - Stubbs equation for vitamin-A acetate in alcohol applies equally to the same compound in *iso*propanol. For vitamin-A alcohol in *iso*propanol he uses the equation—

$$X = 7\left[E_M - \frac{5}{12} E_L - \frac{7}{12} E_H\right]$$

$$= 7[\text{E at } 326\,m\mu - 0{\cdot}4167 \text{ E at } 312\,m\mu - 0{\cdot}5833 \text{ E at } 336\,m\mu],$$

but the U.S.P. Assay[6] of Vitamin A specifies, for vitamin-A alcohol in *iso*propanol, the slightly different equation—

$$X = 7\left[E_M - \frac{3}{8} E_L - \frac{5}{8} E_H\right]$$

$$= 7[\text{E at } 325\,m\mu - 0{\cdot}375 \text{ E at } 310\,m\mu - 0{\cdot}625 \text{ E at } 334\,m\mu],$$

the constants being based on a 23-laboratory co-operative examination of the U.S.P. Standard Vitamin-A acetate *via* the unsaponifiable matter. The recently published 1951 Addendum (Appendix XV, p. 92) to the B.P. 1948 specifies "6/7" correction formulae based on $\lambda_{max.} = 327{\cdot}5\ m\mu$, with $r = 15$ and $s = 10{\cdot}2$, for the esterified vitamin, and on $\lambda_{max.} = 326{\cdot}5\ m\mu$, with $r = 14$ and $s = 10{\cdot}2$, for the alcohol form. Both refer to *cyclo*hexane solutions.

No worker appears to have considered any value for k other than 6/7. From the Morton - Stubbs curve for vitamin-A acetate we can derive numerous equations geometrically no less valid than the "6/7" ones, although, of course, there may be dispositional objections to them. Some examples are—

k	$1/(1-k)$	L, mμ	H, mμ	$s/(r+s)$	$r/(r+s)$
9/10	10	316	336	0·4	0·6
4/5	5	310	341	0·4194	0·5806
3/4	4	308	343	0·4286	0·5714

Morton and Stubbs remark that the "only significance of the ratio 6/7 is that it is empirically appropriate in relation to the wavelength range covered, and to the performance of the spectrophotometer."[3] They had principally in mind high-potency oils, to which the formula originally devised for cod-liver oil was then being applied. There was in practice a special reason for the choice of the 313-mμ fixation point on the absorption curves of cod-liver oil: it seemed least likely to disturb the mathematical assumption of 3-point linearity on the absorption curve of the irrelevant material. Some of the curves obtained by subtraction of the vitamin-A curve from the gross curves of cod-liver oil exhibited small but distinctive peaks at about 305 mμ and 320 mμ associable with conjugated tetra-ene acids, and the trough in between usually reached a minimum at 313 mμ.

A comparison of the results from the standard "6/7" equation with those from the equations corresponding to the sets of constants tabulated above is made later in this paper.

McGillivray[5] uses the correction procedure in principle, but adopts a slightly different geometry that "lends itself to a simpler calculation." Instead of taking fixation points at which the two "shoulder" absorptions are equal (*i.e.*, $a = b = k$), he prefers two points equidistant in wavelength from the point of maximum absorption (*i.e.*, $r = s$). He chooses 310 mμ and 340 mμ for vitamin-A alcohol and the equation is—

$$X = 2\,E_M - 2{\cdot}611\,(E_L + E_H)$$

$$= 2 \text{ E at } 325\,m\mu - 2{\cdot}611\,(\text{E at } 310\,m\mu + \text{E at } 340\,m\mu).$$

Sources of error

Observational errors—

A result that is a function of three E values will clearly have wider limits of error than a result depending on one. To solve the complex problem of how much wider the limits of error are, it must be recalled that what is normally described as "error" in spectrophotometry can be ascribed to several variables, such as the effect of temperature and solvent, and the accuracies of the optical density scale, of the make-up of the test solution, and of the cell dimensions; all these will have in common an exactly parallel influence on the three E values, so that the corrected E value, being a function of the three, will be, as far as these and similar

sources of variation are concerned, in error to exactly the same extent as the uncorrected value. On the contrary, the reproducibility of the *relative* optical densities at the three wavelengths—covered by the term "residual error" in the analysis of variance—has necessarily an inflated influence on the error of the corrected E value. This residual error, therefore, independently affects the three readings, and its inflationary influence can be calculated.

Let us assume that E_M, E_L and E_H have a common coefficient of variation e; in other words, that the standard deviations of the three values are, respectively, $eE_M/100$, $eE_L/100$ and $eE_H/100$. Now, if we write the correction formula as—

$$X = 7[E_M - C_1E_L - C_2E_H],$$

it follows from the theory of errors that the standard deviation of X will be—

$$\frac{7e}{100}\sqrt{(E_M^2 + C_1^2 E_L^2 + C_2^2 E_H^2)}.$$

If we take the constants C_1 and C_2 appropriate for vitamin-A ester in *cyclo*hexane (equation 3, above) and if, for simplicity, we suppose that the irrelevant absorption has zero slope over the three wavelengths (*i.e.*, $E_M - X = E_L - 6X/7 = E_H - 6X/7$), then the coefficient of variation, which is the percentage standard deviation of X, can readily be derived. It is—

$$e \times \frac{7}{17}\sqrt{\left(438\frac{E_M^2}{X^2} - \frac{298}{7}\frac{E_M}{X} + \frac{149}{49}\right)}.$$

This means that the coefficient of variation of a corrected E value is greater than that of the original gross E value by a factor that depends on the amount of irrelevant absorption ($E_M - X$) present at the central wavelength, 328 mμ. If, for example, the amount is 10 per cent., then E_M/X is 1·1 and the factor is 9·2. Some representative evaluations are as follows—

Irrelevant absorption at 328 mμ, %	5	10	15	20
Error factor	8·7	9·2	9·7	10·4

This special case of equality of irrelevant absorption at the three wavelengths is in practice very representative geometrically; it is obvious that within the range of slopes of the irrelevant contribution likely to be encountered the difference between ($E_L - 6X/7$) and ($E_M - X$) will be very close to that between ($E_M - X$) and ($E_H - 6X/7$), and the corresponding differences introduced into the error equation will almost compensate. So that, as far as this point is concerned, we can assume that the factors given above hold for all normal circumstances. The assumption that E_L, E_M and E_H have equal coefficients of variation is not wholly justified; in practice E_L and E_H can be expected to have slightly higher coefficients; this means that the given factors may be very slightly low. It appears, then, that for every 1 per cent. of observational error in a gross reading, an error of the order of 8 to 10 per cent. is to be expected in a corrected reading.

Let us now reconsider the total error of variation of an E value, made up of the residual coefficient of variation e, and the summated variation due to the factors discussed above and whose effect is not magnified by correction: let us call its coefficient d. A duplicate E value from one laboratory will have an effective coefficient of variation of $\sqrt{(d^2 + e^2/2)}$. If three E values are determined and two of them are used to "correct" the third, the resultant corrected E value will have an effective coefficient of variation of $\sqrt{(d^2 + e^2f^2/2)}$, where f is the error factor derived as shown above and whose value is here, say, 9. Typical values for e and d are, respectively, 1·5 and 2; hence, on evaluation it is found that the expected coefficients of variation of results of the type described are 2·3 for normal, uncorrected, E values and 9·8 for corrected E values.

A recent co-operative trial designed to ascertain the error of corrected E values has yielded evidence not inconsistent with this theoretical derivation.[7]

ASSUMPTIVE ERRORS—

Assumptive errors are those introduced by departures from the basic assumptions of the shapes and positions of the two absorption curves, that of vitamin A and that of the irrelevant material.

Let us first consider departures from the assumed characteristics of the curve for vitamin A. Suppose, to begin with, that the true position of the vitamin A is displaced, without distortion, along the wavelength scale. (Such a shift might be artificial, caused by unknown solvent effects or maladjustment of the wavelength scale or, alternatively, natural, in that

the particular esterified vitamin A in the oil might be slightly different chemically or stereoisomerically, and therefore spectrophotometrically, from vitamin-A_1 acetate.) It can be shown that the resulting displacement errors are as follows—

Displacement of vitamin-A curve from assumed position	Corrected E at 328 mμ as percentage of true value
−2 mμ	98·3
−1 mμ	98·7
zero	100
1 mμ	104·1
2 mμ	108·6

It appears that the errors so introduced are small if the vitamin-A curve reaches a maximum at a wavelength one or two millimicrons *less* than that of the assumed maximum, but appreciable if the displacement is in the other direction.

The presence of neovitamin A may appropriately be considered here. Robeson and Baxter[8] have evidence that suggests that the vitamin A of the common fish-liver oils is two-thirds vitamin A_1 and one-third neovitamin A_1, the two forms being equally potent. The

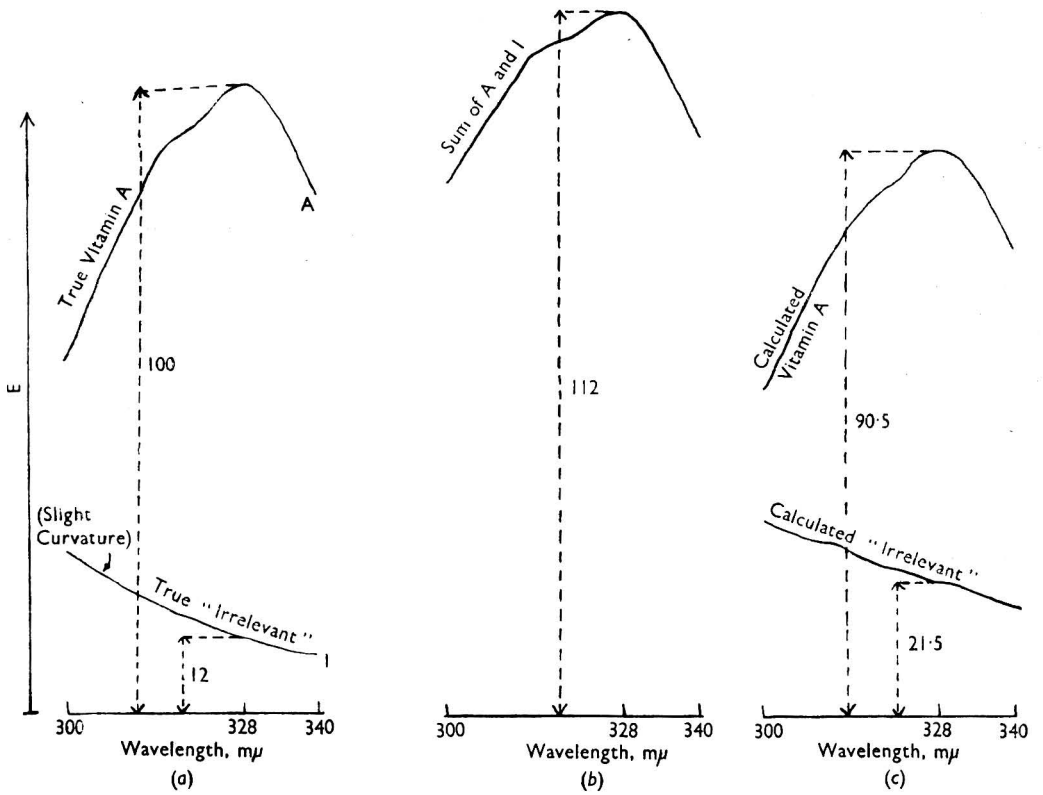

Fig. 2. How a hypothetical mixture of vitamin A and "irrelevant" material responds to 3-point correction of its absorption curve. The 3-point correction of the curve shown at (*b*) partitions it as shown at (*c*)

absorption curve of the neovitamin seems to be similar to that of the ordinary (all-*trans*) material, but shifted 3 mμ further up the wavelength scale, while its absorption at $\lambda_{max.}$ is 10 per cent. lower than that of the ordinary vitamin A at its $\lambda_{max.}$. Suppose that a 2 to 1 mixture of the two forms, uncontaminated and saponified, is assayed according to U.S.P. XIV.[6] At the fixation points the true breakdown would be—

	E at 310 mμ	E at 325 mμ	E at 334 mμ
Vitamin A	59·5	69·4	59·5
Neovitamin A	25·5	30·6	28·1
Total observed	85·0	100·0	87·6

and on applying the correction formula to the observed values the assay would be 93·6 per cent. of vitamin A, instead of the true (biological) value of 100 per cent.

Let us now consider "distortion," *i.e.*, the possibility that the fixation points of the particular sample of vitamin A under test are not quite those assumed in the formula. To set out all the possible kinds and degrees of distortion would be impracticable, but a representative selection is shown in Table I.

TABLE I

EFFECT OF FIXATION-POINT DISPLACEMENT ON E AT 328 mμ (CORR.) OF VITAMIN-A ACETATE IN *cyclo*HEXANE

"Corrected" observed values when the true value is 100

		If the higher 6/7 point, assumed to be at 338·5 mμ, is actually at				
	mμ	336·5	337·5	338·5	339·5	340·5
If the lower point, assumed to be at 313 mμ, is actually at	311	106·0	111·2	117·0	123·3	128·8
	312	98·2	103·4	109·2	115·5	121·0
	313	89·1	94·3	100	106·4	111·9
	314	80·9	86·1	91·9	98·2	103·7
	315	73·5	78·7	84·5	90·8	96·3

Finally, there are possible faults to be reckoned with in the assumption of effective linearity of the curve of the irrelevant material. The term "effective" is used because, as already pointed out, the assumption concerns the E values at the fixation points only, the contour of the curve between and beyond these points being immaterial.

Curvilinearity of the three E values, as of any three points in a plane, can, of course, theoretically cover great distributional variety, but fortunately for the present purpose all distributions can be considered as vertical displacements of the centre point, *i.e.*, values of E at 328 mμ greater or smaller than those interpolated from E at 313 mμ and E at 338·5 mμ of the irrelevant material. It can readily be shown from the fundamental formula that one unit of departure from linearity in this measure (E at 328 mμ) will result in an error of 7 units in the estimation of E at 328 mμ (net) by correction. A hypothetical example is shown in Fig. 2 and in the following—

	E at 313 mμ	E at 328 mμ	E at 338·5 mμ
Vitamin A	85·7	100·0	85·7
Irrelevant	18·5	12·0	9·8
Sum	104·2	112·0	95·5

The three E values for the irrelevant material fall on a curve, which may be considered as a 1·4-unit middle-point displacement from the straight line 18·5, 13·4, 9·8. Correction of the summated E values yields the following analysis—

	E at 313 mμ	E at 328 mμ	E at 338·5 mμ
Vitamin A	77·2	90·1	77·2
Irrelevant	27·0	21·9	18·3
Sum	104·2	112·0	95·5

in which E at 328 mμ for the vitamin-A fraction is 9·9 units (*i.e.*, approximately 7 × 1·4) too low. This is not, it may be mentioned, an exaggerated example: if, as in Fig. 2, the curve through the three points, E at 313 mμ = 18·5, E at 328 mμ = 12·0 and E at 338·5 mμ = 9·8, is plotted, its shape will be found not implausible for the irrelevant material likely to occur in fish-liver oils. Yet there would be a 10 per cent. error if such a mixture were analysed by 3-point correction.

ALTERNATIVE FIXATION POINTS

It is useful at this stage to consider the effect of using correction formulae based on fixation points other than the customary ones, *i.e.*, based on values of k (in formula 2) other than 6/7. Constants for the equations corresponding to $k = 9/10$, 4/5 and 3/4 have already been given; to compare their corrective values to typical curves for oils containing vitamin A the equations have been applied to the absorption data of five samples described and discussed by Adamson *et al.*[7] Of the seven sets of data (one from each of seven independent laboratories) three had for this purpose to be excluded, because one did not include detailed curves and

two included curves insufficiently detailed to permit interpolation of the new fixation points. The mean results from the other four laboratories are set out in Table II, the normal $k = 6/7$ values being included for comparison.

TABLE II

PERCENTAGE OF IRRELEVANT ABSORPTION AT 328 mμ CALCULATED FOR VARIOUS FIXATION POINTS OF FIVE SAMPLES

(Mean values for four laboratories)

	Sample					
k	Concentrate*	Halibut-liver oil	Hake-liver oil	Cod-liver oil	Blend†	Mean
9/10	16·2	21·9	16·7	17·8	15·3 (17·7)	17·6
6/7	10·9	14·2	10·7	12·6	8·0 (11·5)	11·3
4/5	9·5	12·4	11·8	16·6	5·9 (10·1)	10·2
3/4	10·5	13·3	9·3	18·9	7·2 (11·0)	11·8
Mean ..	11·8	15·5	16·5	12·1	8·1	

* A processed high-potency fish-liver oil concentrate.
† A mixture of the first four samples. The bracketed figures are the expected values calculated from the known composition of the blend.

The 80 values condensed into Table II have been analysed for variance with the results shown in Table III.

TABLE III

ANALYSIS OF VARIANCE OF DATA SUMMARISED IN TABLE II

Source of variation	D.F.	Mean square	Variance ratio	Significance (at P = 0·05)
Between laboratories	3	80·3	1·29	not significant
Between samples	4	142·4	2·29	not significant
Between formulae	3	188·6	19·36	significant
Interactions—				
Laboratories × samples	12	62·4	6·40	significant
Laboratories × formulae	9	3·0	<1	not significant
Samples × formulae	12	16·7	1·71	not significant
Laboratories × samples × formulae	36	9·74	1	

From Tables II and III the following inferences can be drawn—

(*i*) There is no significant over-all difference between the four laboratories.

(*ii*) No significant differences are shown between the quantities of irrelevant absorption in the five oils. Nevertheless, there is a tendency for the halibut- and cod-liver oils to give higher, and for the blend to give lower, values than the average.

(*iii*) Laboratories differ in their *relative* placing of the oils: it is only when the results of all laboratories are pooled that these differences are submerged.

(*iv*) The quantities of irrelevant absorption indicated by the four formulae differ. This is almost wholly accounted for by the high "9/10" corrections. But the figures for cod-liver oil can be specially treated: with $k = 6/7$ the yield of irrelevant absorption is markedly less than that given with the other values of k.

This difference does not quite reach significance at P = 0·05, but is still noteworthy.

(*v*) The values calculated from the absorption curves of the blend, and those calculated from the curves of the component oils, appropriately weighted, show discrepancies. Those for $k = 9/10$ are least discrepant.

(*vi*) Except perhaps for cod-liver oil, where there is some chemical evidence for the suitability of the $k = 6/7$ points, there is nothing on which to base a decision as to which of the four sets of fixation points is best.

MECHANISM OF CORRECTION

Analytically, attention can be confined to the E values at the three specified wavelengths. But, having calculated the partition, we may plot the gross absorption curve *in extenso*, subtract from it the vitamin-A curve and so obtain the presumptive curve of the irrelevant material.

Morton and Stubbs have published a number of such curve partitions in illustration of the results of 3-point correction. The irrelevant components usually slope down gradually and unevenly, from the region of low to that of high wavelength. Sometimes, however, the slope is in the other direction and accompanied by slight peaks on the higher wavelength side: the presence of vitamin A_2 ($\lambda_{max.} = 350$ mμ) or of anhydrovitamin A ($\lambda_{max.} = 350$, 370 and 390 mμ) can often be inferred in irrelevant-component curves of this type. The

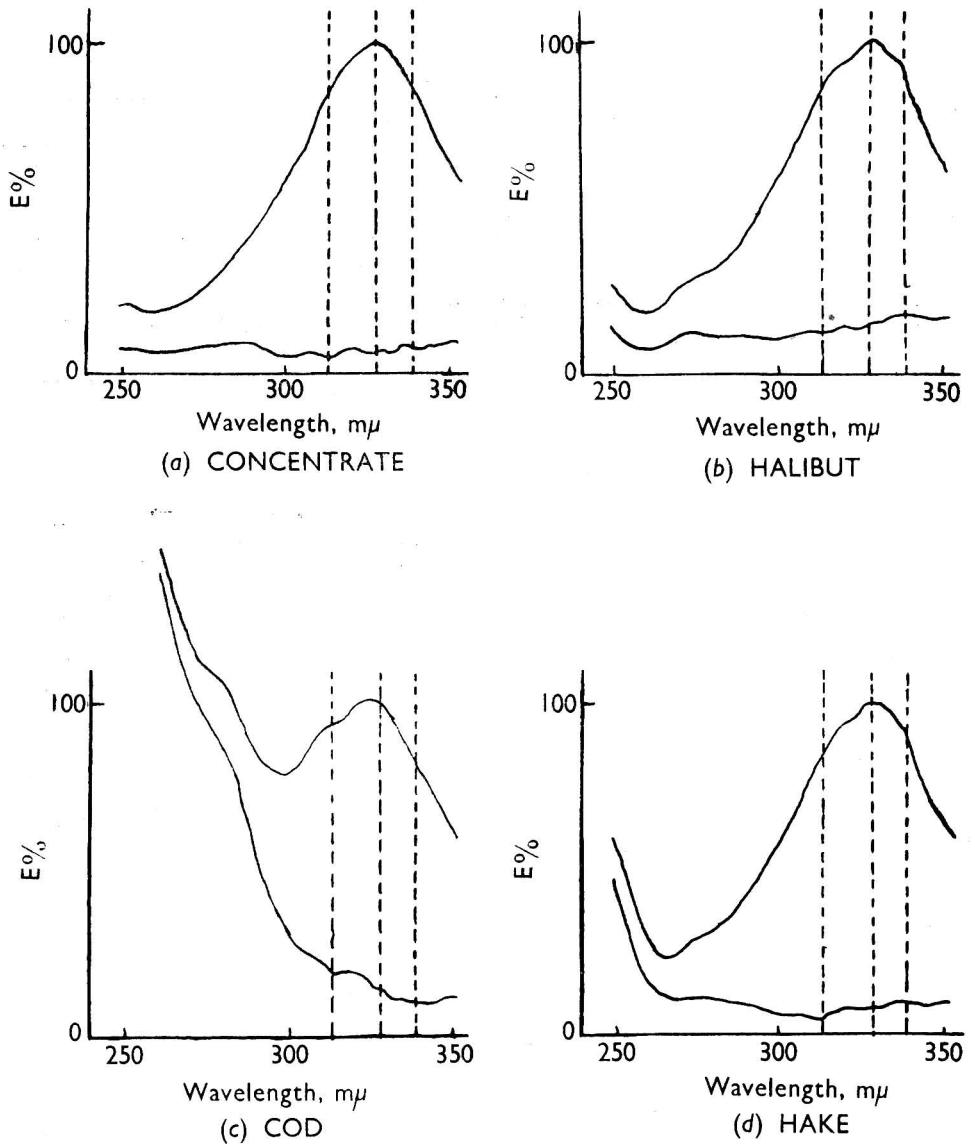

Fig. 3. Absorption curves for the four oils. Upper curves, whole oils; lower curves, "irrelevant" absorptions assumed in 3-point correction at fixation points shown by vertical broken lines at wavelengths of 313, 328 and 338·5 mμ

partitioned curves of cod-liver oil come into a special category because of the low vitamin-A potency of the oil: this means that the ultra-violet absorption of the fatty acids is large in relation to that of the vitamin and plays a big part in shaping the irrelevant-component curve.

In Fig. 3 the absorption curves of the four main oils analysed in the co-operative study reported by Adamson *et al.*[7] have been partitioned on the basis of ordinary ($k = 6/7$) 3-point correction (the fifth sample, a blend, was not included as it was too heavily weighted with

the high-potency concentrate to be illustratively useful in this context). The irrelevant components clearly resemble those described elsewhere.

To understand the mechanism of the correction procedure, it is necessary first to realise that any given gross curve can be partitioned into an infinite number of pairs ranging from

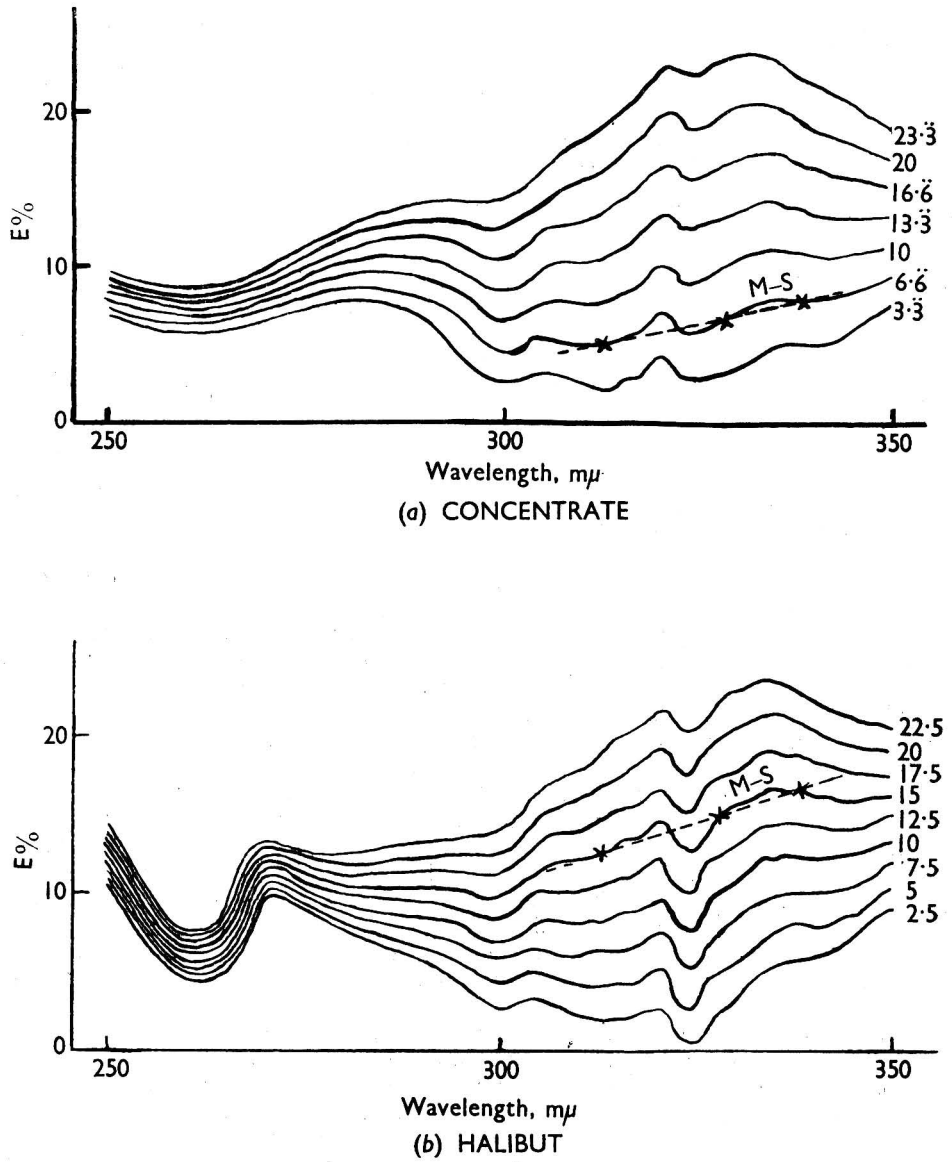

Fig. 4. Theoretical breakdown of whole-oil curves into vitamin-A ester portions (not shown) and "irrelevant" portions

The curves labelled M–S are those assumed in the 3-point correction because the marked points (at 313, 328 and 338·5 mμ) fall on a straight line

$E^{1\%}_{1cm}$ at 328 mμ of whole-oil curve = 100

The figures at the right of the curves indicate the possible percentage of "irrelevant" absorptions at 328 mμ

a certain upper limit (particular to each curve) downward. For example, a curve exhibiting E at 313 mμ = 98·0, E at 328 mμ = 100·0 and E at 338·5 mμ = 79·7 cannot (on the basis of the standard vitamin-A acetate curve) "contain" more vitamin A than that represented by

E at 328 mμ = 93 and E at 313 mμ = E at 338·5 mμ = 79·7 If this maximum is expressed as 93 per cent. of vitamin A and 7 per cent. irrelevant material, this is but the first of an infinite series of theoretical partitions of the original curve ranging down to no vitamin A and 100 per cent. of irrelevant material. Obviously, the last is highly improbable and perhaps the first is unlikely, but somewhere in the range is the true partition, and adjacent to it, and on either side, are numerous theoretical partitions that on graph paper would look equally plausible. Among them is that selected by the correction procedure, *viz.*—

	E at 313 mμ	E at 328 mμ	E at 338·5 mμ
Vitamin A	76·6	89·3	76·6
Irrelevant	21·4	10·7	3·1
Sum	98·0	100·0	79·7

whose only distinctive feature is the linearity of the three points on the lesser curve.

Let us now return to the partitioned curves in Fig. 3. Any one of these lesser curves selected by the correction procedure can be set in its appropriate place in a representative selection of the companion curves whose theoretical existence is discussed above. This has been done for the concentrate and the halibut-liver oil in Fig. 4, where the arbitrariness of the selection made by the correction procedure is reflected in the similarity of the other curves in the vicinity—and even although the extremes in these sets can be rejected as constitutionally improbable, there remains a wide range of possibilities. Another feature emphasised in Fig. 4 is the not inconsiderable discrepancy between the spectroscopic assumption of linearity and the mathematical requirement of linearity of the fixation points, for in between these points the curve is far from smooth.

If these curves are examined further, the similarity in general shape of the two sets is particularly noticeable. Roughly the same absorption peaks and troughs occur, apparently, in the curves of the irrelevant material in two oils of very different natures—one a natural oil and the other a processed concentrate. What the two oils do contain in common is vitamin A and, as the most marked irregularities of the curves of the irrelevant material lie at about the same wavelength as the irregularity near the peak of the vitamin-A curve, the possibility must be considered of some degree of error in the assumption, in the correction method, that the absorption curves of the vitamin-A fatty-acid esters in these oils are identical with the published vitamin-A acetate curve. Graphically it can be predicted that a slight shift of the vitamin-A curves along the wavelength scale would tend to smooth the complementary curves of irrelevant absorption. In Fig. 5, this is done for three representative members of the family of curves of irrelevant absorption from the halibut-liver oil in Fig. 4. Not only does this trial amendment of the assumption smooth the curves, but it materially alters the general slopes. An obvious and simple explanation of the peculiar shapes of the curves as left by the original subtractions would be that the wavelength scale was instrumentally in error. This is unlikely, as the curves are averages obtained from several instruments in different laboratories, all agreeing on the position of $\lambda_{max.}$. Moreover, there is evidence that the absorption curve of the vitamin A chromatographically separated from the concentrate was positioned 2 mμ further up the scale than that of ordinary vitamin-A alcohol.

What could cause the postulated differences between the accepted curves and those encountered in certain—for it is not possible, of course, to say all—oils? First, it is at least conceivable that the acid radicals of vitamin-A esters should slightly influence the contour of the absorption curve, *i.e.*, that the curve for vitamin-A acetate, for instance, was not the same, spectrophotometrically, as that for naturally-occurring esters. Secondly, there may be differences between the absorption curves of the four geometric isomers of vitamin A whose occurrence and characteristics have not yet been fully established. Robeson and Baxter[8] have distinguished between two isomers that occur together in fish-liver oil, and it is significant that they appear to have absorption curves similar in shape but 3 mμ out of step (*vide supra*).

Validity of results

The previous section bears on the question of the validity of results in that it deals with the assumptions fundamental to 3-point correction of vitamin-A absorption curves. What is further needed is some direct evidence, *i.e.*, comparisons between correction estimates and estimates by physico-chemical methods. The literature is not rich in information of this

kind. Morton and Stubbs have discussed three samples of tunny-liver oil with this in mind, but there is not enough range among the samples to give the evidence great strength: it can be concluded that the corrected results are indeed of the same order as the physico-chemical results, but as they are also similar among themselves we cannot be sure that other

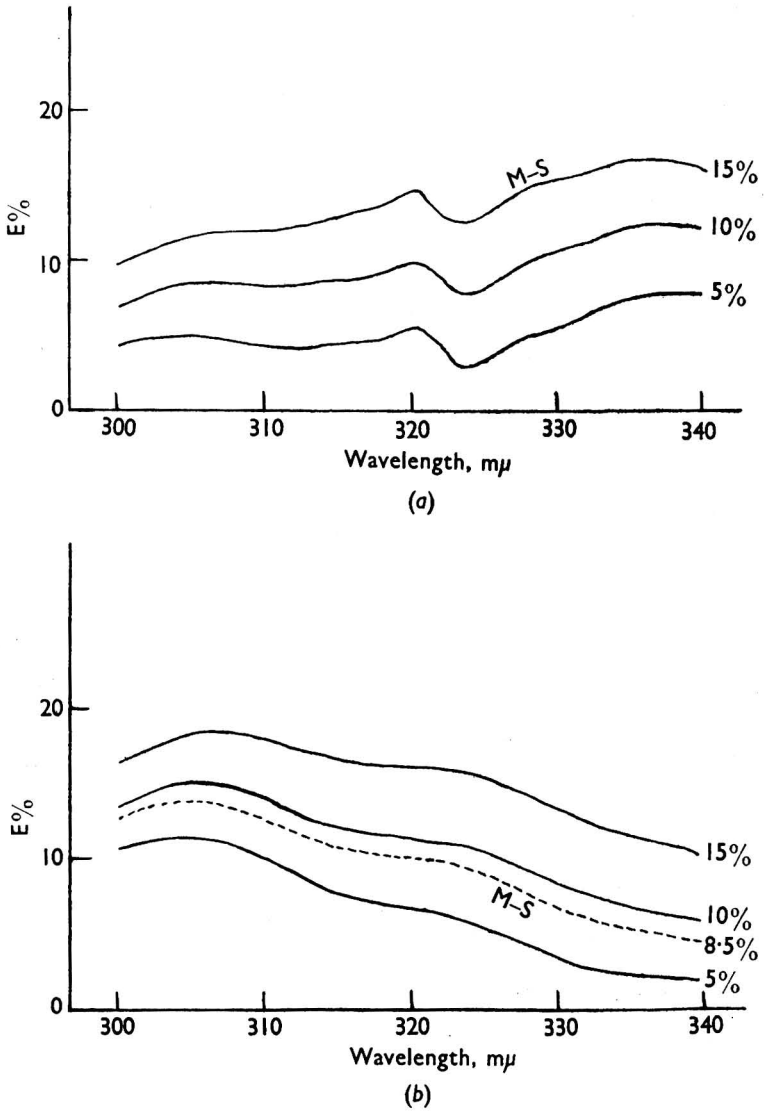

Fig. 5. Effect of assumed wavelength shift on shape of postulated "irrelevant" absorption curves

The curves labelled M–S are those selected by 3-point correction

$E^{1\%}_{1cm}$ at 328 mμ of whole-oil curve = 100

(*a*) Typical trio of the family of Halibut "irrelevant" curves, re-scaled from Fig. 4

(*b*) Comparable curves derived after assumption of a 3-mμ upward shift of the complementary vitamin-A ester curve

samples containing greater or less irrelevant material would respond in proportion. No information on sensitivity therefore emerges.

Data on the 3-point corrected E at 328 mμ values of several fish-liver oils, a distilled ester and vitamin-A acetate have been given by Coetzee,[9] but they contribute little to the

present problem. They turn on biological assays against β-carotene, so that the precision and accuracy are insufficient to show up critical differences. The apparent percentages of irrelevant absorption in the samples are 0·6 per cent. for the acetate, 3·4 per cent. for the distilled ester, and 5·5 to 12·3 per cent. for the fish-liver oils, but the bio-assays correlate almost as well with the gross as with the corrected values (*i.e.*, the coefficients of variation for the gross and corrected "conversion" factors are, respectively, 11 and 9).

More interesting is Morton and Stubbs's collection of assays on cod-liver oil given in their original paper.[1] Each sample was assayed by correction of the whole-oil absorption curve and by determination of the E value *via* the unsaponifiable matter (it has long been observed that most of the irrelevant material in cod-liver oils can be removed by saponification). Morton and Stubb's own tabulation of their results does not make it clear whether the correlation between the two methods is good or bad. In fact it is good, as can be seen from the summary in Table IV.

TABLE IV

CONDENSATION AND REARRANGEMENT OF E AT 328 mμ DATA ON COD-LIVER OILS FROM MORTON AND STUBBS'S TABLE III (*Analyst*, 1946, **71**, 355)

The 20 samples are grouped in five equal sets and arranged in order of percentages of E at 328 mμ due to vitamin A

	Mean E at 328 mμ (gross)	"Irrelevant" E at 328 mμ by 3-point correction, %	"Irrelevant" E at 328 mμ by determination of unsaponifiable matter, %
1st set of 4 samples	1·086	9·1	10·4
2nd "	0·899	14·4	14·5
3rd "	0·832	20·8	19·5
4th "	0·836	23·6	21·6
5th "	0·609	42·5	34·6

These figures suggest that the ultra-violet absorption of cod-liver oil fatty-acids is linear at the fixation points, and therefore, that the method has some validity for this oil. Evidence of a similar kind is needed before the application of the method to other oils can be established. At present its reliability is *sub judice* and its use requires caution.

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January, 1951

Assessment of Adrenocorticotrophic Hormone Activity

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(*Presented at the meeting of the Biological Methods Group on Tuesday, December 19th, 1950*)

A review of attempts to assess adrenocorticotrophic hormone (ACTH) activity biologically is given. The methods discussed include those based on change of adrenal weight and those utilising histological changes within the adrenal cortex. Experiences with the Sayers adrenal ascorbic acid depletion assay are described, together with an account of pituitary blockade by desoxycorticosterone acetate (DOCA) in an attempt to improve the precision of the method.

The use of phosphorus-32 as an index of adrenal function is described. Following a review of indirect assay methods based on changes due to adrenal hormones mobilised by ACTH, attempts to utilise the modification of insulin sensitivity brought about by ACTH are discussed.

Some results of clinical adrenal cortex response tests are reported.

The possibility of the assay of ACTH by using surviving tissue in place of the assay animal is envisaged.

Since it was first recognised that extracts made from the pituitary anterior lobe had an adrenocorticotrophic action, there have been numerous attempts at its qualitative and quantitative assay.

The earliest investigators used the weight increase of the adrenal as a basis for the assay of adrenocorticotrophic hormone (ACTH). Jores and Beck,[1] for example, used the adrenal weight of normal male mice, which are particularly sensitive; Moon[2] used the adrenal weight of 3-day old rats and later of 21-day old rats[3]; Bates, Riddle and Miller[4] used the adrenal weight of 2-day old chicks.

Results of assays of this type, employing intact as opposed to hypophysectomised animals, should be regarded with the greatest reserve, for many factors, such as changes in environmental temperatures or the presence of toxic substances in the extracts used, can influence the weight of the adrenal by mobilising endogenously produced ACTH.

Collip,[5] in his first attempts at standardisation, made use of hypophysectomised animals for assays based on adrenal weight change, but experiments published later from his laboratory by Neufeld[6] showed a complete lack of quantitative correlation between dose and response.

Investigations were carried out a few years ago in our laboratory on hypophysectomised male Wistar rats of 100-g body weight, using ACTH extracts prepared from cattle pituitaries. The results were disappointing, as can be seen from Table I, in which the individual weights of the right adrenals, removed 7 days after hypophysectomy, are recorded below the weights of the left adrenals removed at autopsy after a 7-day injection period. The very large scatter of the results is indicated by the standard deviation of each response; the dose - response curve in Fig. 1 constructed from the data of Table I only emphasises the unsatisfactory nature of the assay procedure. The causes of the large variation in response are at present being investigated in our laboratory and some are discussed below.

Recently, Cortis-Jones, Crooke, Henly, Morris and Morris[7] reported a satisfactory linear relationship between the logarithms of the doses injected and the increase in adrenal weights of hypophysectomised rats. They define one ACTH unit, arbitrarily, as an activity causing a 50 per cent. increase in the mean adrenal weight over that of the hypophysectomised control animals.

Weight maintenance methods developed by Astwood and Tyslowitz[8] and by Sayers, White and Long[9] seemed to yield better results.

For a number of years we used as a method of assay the restitution, produced by ACTH injection, of the sudanophobic zone of the adrenal cortex of hypophysectomised rats, originally described by Reiss, Balint, Oestreicher and Aronson[10] and later by Simpson, Evans and Li.[11] This assay gave a satisfactory linear relationship between the percentage of animals giving positive response (or better still, the probit of the percentage) and the logarithm

TABLE I

INVESTIGATION INTO THE ASSAY OF ADRENOCORTICOTROPHIC HORMONE (ACTH) EXTRACTS WITH 100-g HYPOPHYSECTOMISED MALE WISTAR RATS

Dose of ACTH, mg	Number of animals	Weight of adrenals, mg: Left adrenal after treatment / Right adrenal before treatment	Mean of individual percentage weight difference between right and left adrenals ± S.E. of mean
40	8	9/4 8/5 8/4 5/4 6/3 8/8 10/4 8/6	+ 76 ± 16·5
20	8	10/8 12/7 8/4 6/4 6/5 6/6 12/8 9/7	+ 42 ± 11·3
10	8	11/7 10/6 9/5 8/3 7/4 11/6 12/8 7/6	+ 76 ± 15·1
5	7	5/5 9/8 7/7 7/6 5/4 8/6 5/3	+ 22 ± 8·8
3	6	6/6 6/7 6/5 10/5 8/5 8/6	+ 33 ± 16·0
1	8	6/4 10/7 7/7 7/6 3/4 4/3 4/5 5/6	+ 10 ± 10·5
Controls	8	5/8 4/4 3/3 4/5 4/7 3/6 6/8 4/5	− 22 ± 6·9

of the dose. If one compares the original adrenal weight method illustrated in Fig. 1 with Fig. 2, showing response curves for standard and unknown ACTH preparations by the histological method, the superiority of the sudanophobic zone restitution method is evident without further calculation.

With the histological method 10 to 35 rats per dose and 3 to 4 doses per preparation were required to characterise the response curves, which were found to be very reproducible

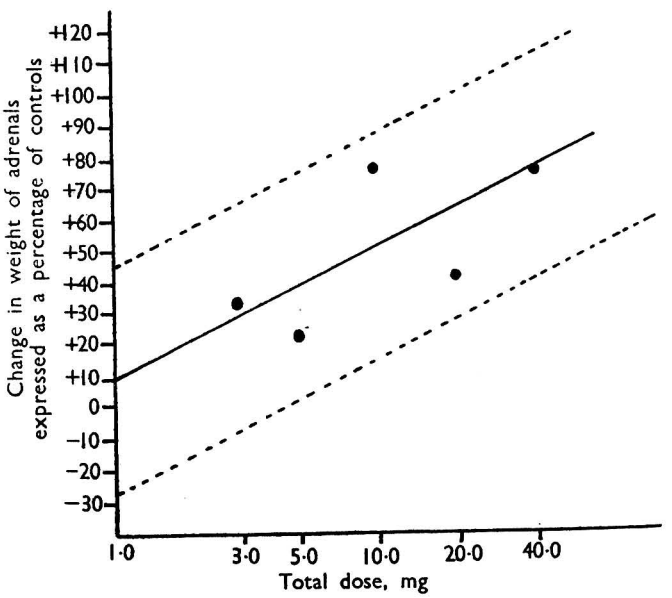

Fig. 1. Relation between log-dose of adrenocorticotrophic hormone and change in weight of adrenals in hypophysectomised rats
The dotted lines are one standard deviation from the line of best fit

and in this respect much superior to those we have since obtained with the adrenal ascorbic acid depletion assay of Sayers and Sayers,[12] to be discussed later. It is a more time-consuming method, however, requiring the maintenance of hypophysectomised animals for 7 to 11 days before assay and for a 3 to 4-day injection period, so that a considerably higher standard of hypophysectomy technique is involved. Though more consistent, it is less sensitive, requiring 50 to 100 times as much material as the adrenal ascorbic acid depletion method.

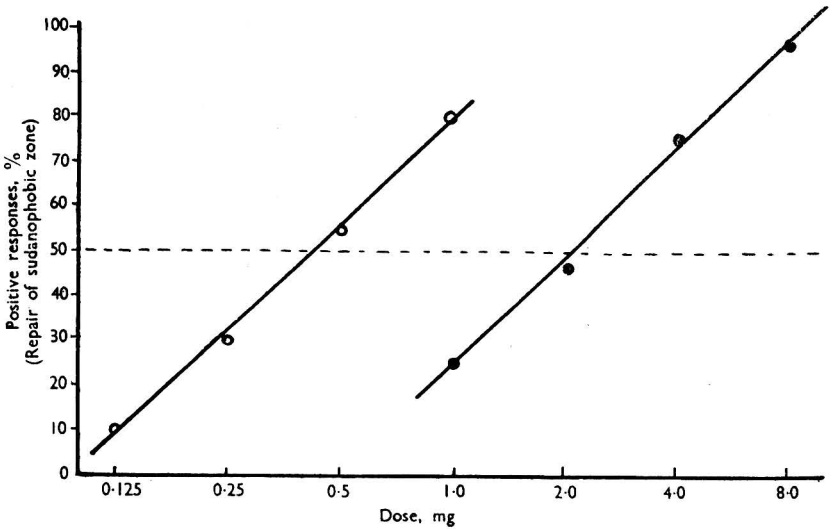

Fig. 2. Comparison between adrenocorticotrophic hormone of unknown strength and laboratory standard by the histological method

Standard ——— ● ——— Unknown ——— ○ ———

Because of the changes observed in the sudanophobic zone, it might have been worth while to follow up more intensively the changes in cholesterol and steroid contents of the adrenals after ACTH treatment, described by Sayers, Sayers, White and Long[13] and later by Carryett, Golla and Reiss.[14] However, interest in this, and for that matter in any other form of assay, was overshadowed by the introduction of the adrenal ascorbic acid depletion assay by Sayers and Sayers.[12]

This method makes it possible to obtain results more quickly, with much less material and with comparatively less labour than does, for instance, the sudanophobic zone restitution assay. It has therefore been of great help in the development of methods for preparing ACTH concentrates. It is carried out as a routine method in our laboratory essentially as described by Sayers, Sayers and Woodbury,[15] with a few alterations. The use of ether anaesthesia throughout, the choice of the femoral vein as the injection site, a personal preference for the right adrenal as the control and the use of an electrotitrimetric method for ascorbic acid determinations are the only important differences between our technique and that of the originators.

Table II summarises the results of some of the assays carried out in our department by this method. It shows the number of rats used, the slopes of the response curves for standard and unknown, the value of χ^2 (the test for heterogeneity between the two lines), the standard deviation of the assay and the standard error of M (the logarithm of the ratio of potencies) for P = 0·95 and the approximate limits of error calculated from the standard error of M. The first three entries refer to separate one-day (3 + 3) assays comparing the same preparations. The fourth entry gives the evaluation of the combined results of the three assays, which does not show a significant improvement in precision over that of the individual assays. On the other hand the assay carried out on September 27th with only 30 animals gave a result of far greater precision.

The irregular nature of the response curves indicated by Table II caused much misgiving when we first began to use this method of assay. It appeared that several hundreds of animals would be required to obtain satisfactory limits of error.

The occurrence every now and then of an assay completely in agreement with results published by Sayers, Sayers and Woodbury,[15] by Morris and his co-workers,[7] by Prunty[16] and by other authors, in that reasonable limits of error were obtained with approximately 30 animals, led us to investigate as fully as possible the causes for the apparently chance variation of our results.

Over the question of the suitability or otherwise of the strain of Wistar rats bred in our colony we received considerable help, obtaining, through the courtesy of Dr. Morris, Wistar rats of his own strain. We hope to breed from these animals and compare the results with those obtained on our own rats, which are partly derived from the original Glaxo strain and partly from the strain bred by Dr. Paterson in Porton. That the solution lies solely in the use of a different strain of rats seems unlikely, for it does not explain why our own animals sometimes respond as satisfactorily as those of other workers.

TABLE II

SUMMARY OF RESULTS OF SOME ASSAYS OF ACTH BY THE ADRENAL ASCORBIC ACID DEPLETION METHOD

Date of assay	Number of rats	Slope of log-dose - response curves		χ^2	Standard deviation of the assay	Standard Error of M* (P = 0·95)	Approximate limits of error, %
		Standard	Unknown				
21.4.50	44	58·2	63·1	0·059	± 26·7	0·2634	± 64
26.4.50	40	91·3	95·6	0·037	± 33·1	0·2300	± 56
5.5.50	47	144·3	117·4	3·610	± 31·0	0·1355	± 32
21.4.50 26.4.50 5.5.50	131	104·5	85·2	3·210	± 32·3	0·1331	± 31
10.5.50 12.5.50	78	101·0	79·1	2·0	± 24·9	0·1701	± 40
17.5.50	16	149·0	139·0	0·103	± 22·0	0·1510	± 35·5
27.9.50	30	116·3	114·3	0·077	± 13·7	0·0851	± 19·5
9.11.50	23	125·5	142·8	0·049	± 22·6	0·1448	± 34
14.11.50	25	116·4	78·1	0·100	± 22·2	0·2028	± 48·5
8.12.50	26	84·25	86·25	0·008	± 25·5	0·2455	± 54·5
Log-dose - response curve for 60-g hypophysectomised rats							
7.6.50	9	124·6	—	—	± 34·9	s/b = 0·280	

* M is the logarithm of the ratio of potencies.

It would appear more likely that the variation is due to the fact that no other anterior pituitary hormone can be so readily mobilised endogenously as ACTH. The ascorbic acid depletion index shows how even small manipulations on an intact animal, *e.g.*, anaesthesia, injection of test material or even changes in environmental temperature, can bring about a mobilisation of ACTH with a consequent fall in adrenal ascorbic acid concentration. It is obvious that such stresses as fights with other rats and draughts in the animal house can occur in the life of a growing rat, mobilising temporarily increased amounts of endogenous ACTH with its prevailing effect upon the adrenal cortex. This might be the sole explanation for the variation in adrenal weight per unit body weight found among the members of a large rat colony. The point is important, because the dose injected is calculated per 100 g of body weight and may therefore be called upon to influence adrenals of very different weights. Obviously the younger the rat the fewer the occasions for irreversible changes in the adrenal cortex. This consideration, together with the observation that it is easier to induce weight changes in the adrenals of younger than of older rats, led us to use 60-g hypophysectomised rats for assay purposes; the result of one rather promising experiment is included in Table II. The precision obtained is comparable with that of most of the assays on older animals, although the scatter of the responses is still unsatisfactory. It would be preferable to use even younger rats, but in a 40-g animal the stress of hypophysectomy is very much greater than in older ones. For this reason we studied the known effect of desoxycorticosterone acetate (DOCA) in blocking the pituitary function of ACTH mobilisation, with a view to using animals pre-treated in this way for assay purposes.

In Table III is shown the effect of injecting DOCA into 40-g rats on the endogenous production of ACTH by the pituitary, following stress. The stress applied consisted in anaesthesia with ether and the rapid removal of one adrenal. One hour later the animals were killed, the second adrenal was removed and the concentrations of ascorbic acid in the adrenals were compared.

TABLE III

EFFECT OF DESOXYCORTICOSTERONE ACETATE (DOCA) ON THE ENDOGENOUS PRODUCTION OF ACTH BY THE PITUITARY, FOLLOWING STRESS

Treatment		Number of rats	Concentration of ascorbic acid in right adrenal, mg per 100 g, ± S.D.	Mean difference in ascorbic acid concentration between right and left adrenals, mg per 100 g, ± S.D.
Material injected and dose	Time before stress, hours			
No injection	—	5	343 ± 38·1	− 73 ± 23·4
1 ml of arachis oil	18	4	385 ± 95·4	− 63 ± 26·2
1 mg of DOCA in 1 ml of oil	18	4	337 ± 18·4	− 94 ± 4·2
10 mg of DOCA in 1 ml of oil	18	5	386 ± 50·5	+ 62 ± 39·9
10 mg of DOCA in 1 ml of oil	18	4	385 ± 50·2	− 5 ± 10·9

The response to the stress, in the un-pre-treated control group, is indicated by the considerable drop in concentration of ascorbic acid in the second adrenal. One millilitre of arachis oil or 1 mg of DOCA in 1 ml of arachis oil injected 18 hours before stress did not change this reaction. However, 10 mg of DOCA given 18 hours before stress completely prevented depletion of ascorbic acid. It may be pointed out that it is necessary to treat with DOCA at least 18 hours before assay, as the injection is itself a stress: 4 hours after such an injection the concentration of ascorbic acid in the adrenal is still considerably reduced, so that no further decrease can be achieved by a further stress. Only after 18 hours at the earliest is the ascorbic acid concentration sufficiently high to allow the effect of another stress to be recorded efficiently. Table IV shows the reduction of the adrenal ascorbic acid by different doses of ACTH in 40-g male Wistar rats pre-treated with 10 mg of DOCA 18 hours before assay. The scatter of results is still considerable, and we are investigating the possibility of improving the technique of pituitary block.

TABLE IV

REDUCTION OF ASCORBIC ACID IN THE ADRENALS BY DIFFERENT DOSES OF ACTH INJECTED INTO 40-g RATS PRE-TREATED BY INJECTION OF 10 mg OF DOCA 18 HOURS BEFORE ASSAY

Dose of ACTH, μg per 100 g of body weight	Number of rats	Difference between amount of ascorbic acid in right and left adrenal, mg per 100 g, ± S.D.	Log-dose - response curve characteristics
1·0	3	41 ± 29·4	Slope (b) = 60·3
2·0	4	51 ± 30·7	Standard deviation (s) = 26·15
4·0	5	76 ± 20·0	Index of precision (s/b) = 0·433

The difficulties encountered in the adrenal ascorbic acid depletion assay will be found in any assay method with rats, owing to the extreme ease in mobilising endogenous ACTH during the animal's lifetime. It is in our experience particularly true of adrenal repair tests or those based on hypertrophy of the adrenals, when a further complication occurs because the glands are continuously atrophying during the three weeks following hypophysectomy and the weight increases achieved after treatment are at the best a result of the action of the several factors concerned.

It might therefore be advantageous instead to investigate the biochemical changes that are the primary basis of the growth phenomenon. Previous investigations by Reiss[17] and by Reiss, Druckery and Fischl[18] on the ovary showed up to 100 per cent. increases in oxygen consumption and glycolysis 1 hour after injection of gonadotrophic hormone, long before any growth could be seen. The adrenal cortex[19] also shows similar increases in oxygen consumption and glycolytic processes soon after injection of ACTH. The methods used in such investigations are too cumbersome for application to routine assay procedure, and the

dose–response relationships are not satisfactory. It is therefore simpler to investigate phosphorylation processes in the adrenal cortex, particularly as Gemzell[20] has shown that ACTH increases phosphorylation rate in the adrenal.

Changes in the uptake of phosphorus-32, soluble in trichloro-acetic acid, by the adrenal, as an arbitrary index of phosphorylation rate, were investigated in numerous experiments by Reiss and Halkerston.[21] The effect of endogenously mobilised ACTH was studied in animals exposed to cold, and increases of several hundred per cent. in uptake of phosphorus-32 by the adrenals were recorded after exposure for 1 hour. This change was not shown by hypophysectomised animals. It was also interesting to note that new-born animals at the age of three and eight days are already able to mobilise considerable amounts of endogenous ACTH. Table V shows that even the removal of litter mates from the warm environment of the mother and leaving them at room temperature resulted in a considerable increase in the uptake of phosphorous-32 by the adrenals. Removal to a cool room still further enhanced this reaction. These changes in the uptake of phosphorus-32 by the adrenal parallel the ascorbic acid depletion known to occur under similar conditions.

TABLE V

EFFECT OF ENVIRONMENT ON ADRENAL UPTAKE OF PHOSPHORUS

New-born rats, litter mates, injected with 6 μc of phosphorus-32 intraperitoneally and killed 60 to 80 minutes later. Trichloro-acetic acid extracts of adrenals measured in a liquid counter.

Litter number	Rat number	Treatment	Impulses per 100 mg of adrenal
1 (3 days old)	1	Left with mother	550
	2	Kept 1 hour at room temperature	850
	3	Kept 1 hour in cold room	1165
2 (8 days old)	1	Left with mother	465
	2	Left with mother	455
	3	Kept 1 hour at room temperature	660
	4	Kept 1 hour in cold room	830
	5	Kept 1 hour in cold room	1940

Recently we have taken advantage of the fact that during routine use of the ascorbic acid depletion assay it is possible to introduce by intravenous injection, after the removal of the control adrenal, a dose of phosphorus-32 together with the ACTH test solution. An aliquot of the metaphosphoric acid filtrate from the left adrenal contains a greater amount of phosphorus-32 than that of control animals, and Table VI shows that promising dose-response relationships are obtained between the percentage of the phosphorus-32 taken up by the adrenal and the dose of ACTH injected.

TABLE VI

DOSE-RESPONSE RELATIONSHIP BETWEEN PHOSPHORUS-32 TAKEN UP BY ADRENALS AND DOSE OF ACTH INJECTED

Standard preparation of ACTH			Unknown preparation of ACTH		
Dose in μg per 100 g of body weight	Number of rats	Percentage of dose of ^{32}P taken up by left adrenal	Dose in μg per 100 g of body weight	Number of rats	Percentage of dose of ^{32}P taken up by left adrenal
0·5	5	0·049	0·75	4	0·061
0·25	5	0·037	0·375	5	0·052
1·0	5	0·054	1·0	4	0·058
0·5	5	0·041	0·5	5	0·0475
0·25	2	0·031	0·25	7	0·0435

Such changes in oxygen consumption and uptake of phosphorus-32 are by no means confined to the adrenal cortex. The turnover rate for phosphorus-32 is increased in most growing tissue, particularly tumours. Similarly, changes in the ascorbic acid concentration of the adrenal need not necessarily be connected with the endocrine function of the adrenal cortex; one should therefore be somewhat reluctant to use this index as a basis for the biological

assay of preparations having adrenocorticotrophic action. The use of the adrenal ascorbic acid depletion assay before the mechanism of the depletion is known reminds one of previous attempts to standardise the hormonal activity of pituitary posterior lobe extracts, prepared for their clinical pressor, oxytocic and antidiuretic activities, by their action on the melanophores of frogs.

Indirect tests, based on the quantitative action of adrenocortical hormones mobilised by exogenous doses of ACTH, might therefore be more satisfactory from the point of view of the physiologist and the clinician. Several workers have shown that animals may become more sensitive to certain injurious factors after hypophysectomy and have indicated the possibility of increasing their resistance by treatment with ACTH or adrenocortical hormones.

Tyslowitz and Astwood,[22] for instance, reported the effect of ACTH on the resistance of hypophysectomised rats to low environmental temperatures and later reported[23] the increased resistance of these animals to low environmental pressures after treatment with ACTH. Li, Simpson and Evans[24] described the action of ACTH in increasing resistance to cold, starvation and anoxia, while Reiss, MacLeod and Golla[25] showed that rats treated with ACTH exhibit increased resistance to secondary shock symptoms (rapid fall in oxygen consumption and body temperature, rise of blood haematocrit value and fall in blood sodium) induced by intraperitoneal injections of hypertonic glucose solution. Recently, Buttle[26] reported on the increased resistance of guinea-pigs to histamine after ACTH administration, and Prunty[16] has based an assay on the antagonistic effect of ACTH to wound-healing in mice. All of these actions can, of course, be used as a basis for quantitative bio-assay as soon as standardised conditions can be worked out.

Recently we have investigated the effect of ACTH in increasing the resistance of mice to insulin. Table VII shows the results of two such experiments where groups of about

TABLE VII

EFFECT OF ACTH ON SENSITIVITY OF MICE TO INSULIN

Dose of ACTH, given 1 hour before injection of insulin, mg per animal	Number of mice	Percentage of mice reacting 2 hours after injection of insulin
(*a*) 0·2 *units of insulin injected subcutaneously*—		
nil (controls)	20	55
0·1	19	32
0·4	20	25
1·0	20	20
(*b*) 0·045 *units of insulin injected subcutaneously*—		
nil (controls)	30	93
0·1	18	39
1·0	22	4·5

20 mice were injected with ACTH 1 hour before insulin administration. In the first experiment the occurrence of insulin coma or death 2 hours after 0·2 units of insulin was taken as a criterion of insulin action, while in the second experiment the criterion was the percentage of animals showing at least paralysis of the hind limbs 2 hours after a lower insulin dose. In both experiments the degree of protection increased with dose. The results appear encouraging, particularly since it should be possible for all laboratories with well-established facilities for mouse insulin tests to apply them to the bio-assay for ACTH. The method could doubtless be considerably improved in precision and sensitivity by pre-treatment of the animals with DOCA, which increases the insulin sensitivity considerably by blockage of the pituitary anterior lobe.

A discussion of the assessment of ACTH activity would not be complete without reference to the response of human subjects to ACTH administration. Specific response tests for adrenocortical and adrenocorticotrophic efficiency worked out by Forsham, Thorn, Prunty and Hills,[27] Pincus[28] and other authors are becoming more and more a routine procedure in pathological laboratories.

It is at once apparent when reviewing the different indirect methods of assay that different adrenocortical products are concerned in such effects as resistance to cold, low atmospheric pressure, starvation, insulin and secondary shock symptoms. This becomes still more obvious with the responses of different human subjects to a single injection of ACTH;

even if the response tests are restricted to three secondary actions, *viz.*, decrease in circulating eosinophils, increase in uric acid - creatinine ratio and increase in 17-ketosteroid excretion after ACTH administration, very different reaction types can be found among human subjects. In a large proportion of the tests the results show an eosinophil count decreased by at least

TABLE VIII

RESPONSE OF OVER 400 PATIENTS TO INJECTION OF DOSES OF ACTH AND TO STIMULATION OF ENDOGENOUS ACTH BY INJECTION OF GLUCOSE AND BY ELECTRIC SHOCK TREATMENT

Treatment	Response measured by: Circulating eosinophils, %	Uric acid - creatinine ratio, %	17-Ketosteroid excretion, %
Glucose injection	41	34	9
Low ACTH dose, 15 to 25 mg ..	76	58	18
High ACTH dose, 50 mg	95	62	26
Electric shock	100	85	35

30 per cent., an increase in uric acid - creatinine ratio by at least 25 per cent. and an hourly 17-ketosteroid excretion increased by at least 20 per cent. In other tests, however, only the eosinophil count falls, while the other two indices do not alter. Other cases show an unchanged eosinophil count, one or both of the other components showing significant increases. The fact that an increase in the ACTH dose produces a full response, when the low dose failed to do so, suggests that different independent mechanisms are concerned, some of which are

TABLE IX

INDIVIDUAL RESPONSES OF PATIENTS TO INJECTION OF DOSES OF ACTH AND TO STIMULATION OF ENDOGENOUS ACTH BY ADMINISTRATION OF GLUCOSE AND BY ELECTRIC SHOCK TREATMENT

		Response to small dose of ACTH (15 to 25 mg)			Response to higher dose of ACTH (50 mg)		
Patient	Age	Eosinophils	Uric acid - creatinine ratio	17-Keto-steroid excretion	Eosinophils	Uric acid - creatinine ratio	17-Keto-steroid excretion
1	25	+	0	0	+	0	0
2	42	0	+		+	+	0
3	26	0	0	0	+	+	+
4	28	+	+	+			
5	30	+	+	+			
6	32	+	+	+	+	+	0
7	42	+	0	+			
8	40	+	+	+			
9	26	+	0	+			
10	40	+	0	0	+	+	0
11	46	+	0	0			

		Response to glucose (150 g administered orally)			Response to electric shock treatment		
Patient	Age	Eosinophils	Uric acid - creatinine ratio	17-Keto-steroid excretion	Eosinophils	Uric acid - creatinine ratio	17-Keto-steroid excretion
1	25	+	0	0	+	+	+
2	42	0	0		+	+	+
3	26	0	0	0	+	+	0
4	28	0	0	0	+	0	0
5	30				+	+	0
6	32	0	0	0	+	+	0
7	42	0	0	0	+	+	0
8	40	+	0		+	0	0
9	26	0	+	0	+	+	0
10	40	+	0	+	+	+	0
11	46	+	0	+	+	0	+

disturbed under pathological conditions. The threshold becomes increased and, therefore, the particular response is absent at low dose levels. In Table VIII are shown the results of response tests performed on over 400 patients[29] each of whom received in turn 25 mg of ACTH, 50 to 100 mg of ACTH, then, as mobilisers of endogenous ACTH, 150 g of glucose by injection, and finally electric shock treatment. From this table the general pattern of the response would appear to be an enhanced reaction with increase in the strength of stimulus. That this is only true for the low and high ACTH dose is shown by the individual responses set out in Table IX. Patients 1 to 3, for example, react according to the over-all picture in Table VIII, while patients 4, 5, 6 and 8 show a complete response to even the low ACTH dose, though not to the greater stimulus of electric shock. In other patients it would appear that different adrenal cortex responses are evoked by the different stimuli.

Many of the results are difficult to explain and the whole picture remains obscure, but it seems difficult to avoid the conclusion that there are different adrenocorticotrophic components.

A review of all the bio-assays and possibilities of bio-assay for ACTH emphasises the shortcomings of purely biological methods. It would seem that it will not be possible to overcome all the inherent difficulties of assays based on living animals with their individual demands for adaptation. This demand is a prominent feature of the whole life of the animal before being taken into the assay experiment and is concomitant with the mobilisation of adrenal cortex hormones. In other words, it would be a great advantage if one could dispense with the assay animal altogether and investigate the influence of ACTH and different ACTH fractions on adrenal cortex tissue *in vitro*.

As a step in this direction we are trying to determine the amount of "cortin-like" substances (*i.e.*, neutral lipoid-soluble reducing substances) produced by adrenal cortex tissue *in vitro* and to find how this production can be influenced by ACTH added to the medium. The results obtained so far have proved encouraging and we shall report more fully when the experimental conditions have been stabilised.

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DISCUSSION

DR. W. PERRY said that variations introduced by stress could be reduced by animal training. He thought it might be possible to use non-hypophysectomised animals for assay purposes on a basis of the modification of their immunity responses.

DR. L. J. HARRIS enquired whether the electrometric method used by the authors for the estimation of ascorbic acid was a photo-electric or a potentiometric method and, if the latter, the nature of the electrode used.

DRS. HALKERSTON and REISS said, in reply, that they had not tried any of the possibilities suggested by Dr. Perry, but they did not think that any advantage could be gained by their use. The method used or ascorbic acid estimation was potentiometric, with clean platinum electrodes and a small potential applied across them for a dead-stop end-point. The oxidising agent was 2:6-dichlorophenolindophenol

The Adrenal Ascorbic Acid Depletion and Adrenal Repair Methods for the Bio-Assay of Adrenocorticotrophic Hormone

BY C. J. O. R. MORRIS

(*Presented at the meeting of the Biological Methods Group on Tuesday, October 24th,* 1950)

The methods for the bio-assay of adrenocorticotrophic hormone (ACTH) are discussed. An example of the adrenal repair methods is included in the form of a detailed description of the technique used in the author's laboratory on male Wistar rats, 35 to 42 days old.

Methods that use histological evidence of repair as the criterion of ACTH activity are discussed; these are much more sensitive than those involving adrenal weight.

The adrenal ascorbic acid depletion method is described and discussed, and modifications are mentioned.

THE first methods to be used for the bio-assay of the pituitary adrenocorticotrophic hormone (ACTH) were based on increase in size of the adrenals after subcutaneous injection of the hormone. As the technique of hypophysectomy in the rat became more generally practised, it was soon realised that removal of the experimental animal's endogenous supply of ACTH was essential for reliable assay. This point cannot be too strongly stressed. The use of immature normal animals for ACTH assay by many workers has led to the publication of much erroneous information. Investigation in our own laboratory has indicated that, even under the most carefully controlled conditions, reliable results cannot be obtained on the intact animal. The point also arises in the assessment of the effect of ACTH on the human subject and must always be borne in mind in such experiments.

ADRENAL REPAIR METHODS—

The technique of the adrenal repair methods for the assay of ACTH on the hypophysectomised rat is broadly as follows. The animals are hypophysectomised at 5 to 6 weeks old and a period of 7 to 14 days is allowed for the adrenals to atrophy. The test material is then injected, usually twice daily for 3 days, and the animals are killed on the day following the last injection. Adrenals and testes are dissected free of extraneous tissue and weighed. The weight of the testes provides a criterion of completeness of hypophysectomy, although it must be remembered that, in the assay of crude preparations, the presence of gonadotrophic hormones may lead to an increase in weight of the testes.

As an example the technique as practised in our laboratory will be described in detail. Male Wistar rats, 35 to 42 days old, are used. The animals are bred in our own colony, as it appears that strain and uniform maintenance conditions from birth are essential for reproducible results. In particular, adequate nutritional status is most important. Animals of this age and strain should weigh 90 to 110 g, and rats outside this range should not be used. The animals are hypophysectomised by the parapharyngeal approach. They are kept for

10 to 14 days in a ventilated enclosure thermostatically maintained at 25° C. The test material is injected subcutaneously in a volume of 0·5 to 2·0 ml twice daily for 3 days. Groups of 4 or 5 animals per dose level are used. A similar group may be injected with saline as a control. The animals are killed on the fourth day, and the adrenals are carefully dissected free of extraneous tissue, quickly dried with filter-paper and weighed to 0·1 mg. The testes are also dissected, dried and weighed to 5 mg. Under our conditions a combined testis weight of less than 500 mg is taken as the criterion of completeness of hypophysectomy. The adrenal weight is expressed as milligrams per 100 g of body weight at the time of the

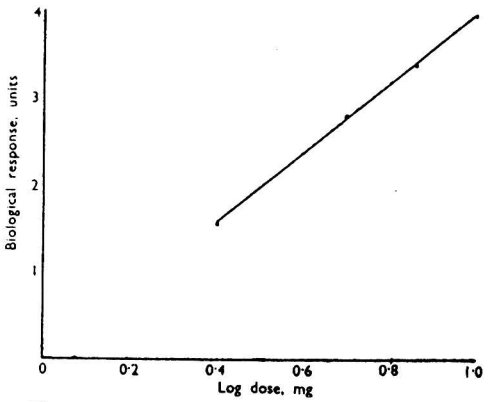

Fig. 1. Assay of adrenocorticotrophic hormone by adrenal repair method. Preparation AH44

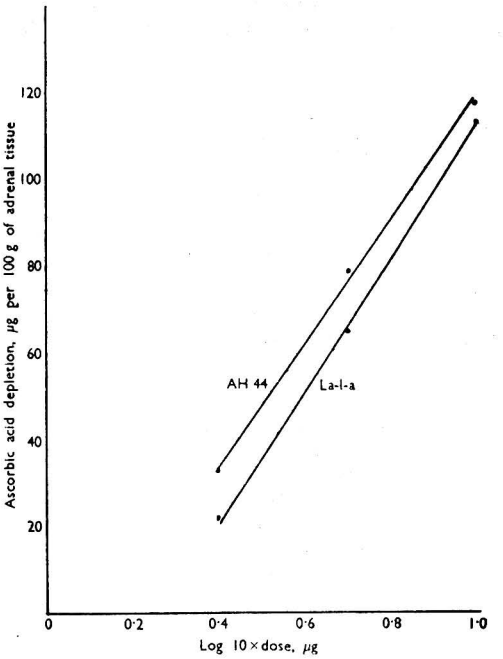

Fig. 2. Assay of adrenocorticotrophic hormone by adrenal ascorbic acid depletion method

first injection. An arbitrary unit has been taken as equivalent to a 50 per cent. increase in mean adrenal weight over that of the controls. Results for a typical assay are shown in Fig. 1. It will be seen that there is a satisfactory linear relation between log-dose and biological response.

The preparation used was slightly more active than Armour Standard La–l–a (× 1·18), so that 1 mg of such a preparation gives a response of 0·4 unit. The useful range of the method is between 1 and 4 units ($P < 0·05$).

It will be seen that under carefully controlled conditions the method is at least as accurate as any other and has the advantage of simplicity. It is, however, rather insensitive and requires 4 days, compared with 1 day for the adrenal ascorbic acid depletion method.

Various modifications of the adrenal repair method have been suggested. Collip[1] used repair of the remaining adrenal in the hypophysectomised unilaterally adrenalectomised rat, the animal serving as its own control. In view of the marked difference in size between the adrenals of a rat, this procedure has little to recommend it. Sayers, White and Long[2] used three daily intraperitoneal injections of test material. Comparison of this method with our own of two subcutaneous injections revealed little difference.

METHODS MAKING USE OF HISTOLOGICAL EVIDENCE OF REPAIR—

In a somewhat different category are the methods that use histological evidence of repair as the criterion of activity. Reiss, Balint, Oestreicher and Aronson[3] first described a disappearance of lipids from the greater part of the adrenal cortex following hypophysectomy. The remaining lipids in the zona glomerulosa become irregular in distribution. Simpson,

Evans and Li[4] based an assay method on the ability of ACTH preparations to reverse these changes. A total of 25 μg of a preparation roughly equivalent to Armour Standard injected intraperitoneally, 6·25 μg once daily for 4 days, was sufficient to produce the first detectable signs of repair. The assay is thus much more sensitive than the methods making use of adrenal weight. In our own experience the method is somewhat difficult to carry out, owing to lack of sharpness at the end-point, and in any event, in common with most "all or none" bio-assays, it requires a large number of animals for precision.

Another method of this type is the adrenal maintenance method. Here the test material is injected for a period of 14 days, beginning the day after hypophysectomy. The unit is defined as the least quantity of the preparation sufficient to maintain the adrenals at a weight equal to those of normal controls. A total dose of 350 μg of a preparation about equal to Armour Standard is adequate. The method is thus roughly ten times as sensitive as the repair test, but requires a large number of animals. It has been described in detail by Sayers, White and Long[2] and by Simpson, Evans and Li[4]; we have ourselves had no experience with it.

ADRENAL ASCORBIC ACID DEPLETION METHOD—

The method of ACTH assay most generally used at present is the adrenal ascorbic acid depletion method. This is based on the observation of Sayers, Sayers, Liang and Long[5] that a single injection of ACTH causes a rapid fall in the ascorbic acid and cholesterol contents of the adrenals. This process is now interpreted as being the first stage in the synthesis of adrenocortical hormones, for it is soon followed by the increase in liver glycogen typical of treatment with certain of these steroids. As this mechanism is set in action by any stress stimulus, it is essential to hypophysectomise the test animal. Long *et al.*[5] showed, however, that the response was diminished and finally disappeared a few days after hypophysectomy, and it is now customary to use the animals the day after operation. Sayers and Sayers[6] and later Sayers, Sayers and Woodbury[7] developed the method into an extremely sensitive, rapid and specific bio-assay method for ACTH. We use rats of 90 to 110 g body weight. It appears that in this method the strain is extremely important, and many strains are entirely unsuitable owing to insensitivity and irregularity of response. The literature shows that some of the standard curves quoted have a very low slope, and the common strains of rat appear to fall into two groups in this respect. We have used our own albino Wistar strain and are very fortunate in having a type of rat of extremely high sensitivity. The animals are hypophysectomised by the usual method; as evidence of completeness of hypophysectomy is difficult to obtain, care should be taken to use only those animals in which the operator is satisfied that removal is complete. The method thus requires a high standard of operative technique. The following day the animal's left adrenal is removed under anaesthesia, dissected free of extraneous tissue, dried, weighed to 0·1 mg and immediately homogenised in a modified Potter - Elvehjem homogeniser with the solvent used for extraction. The test material is then injected intravenously in the proportion of 0·2 ml per 100 g of body weight. Sayers, Sayers and Woodbury[7] used the tail vein, but we have preferred the jugular vein for this purpose. Exactly one hour after injection, the second adrenal is removed under anaesthesia, dissected, dried, weighed and homogenised as before. The animal is killed and the sella turcica examined under a low power microscope for remnants of the pituitary. The choice of anaesthetic is of importance. Sayers, Sayers and Woodbury[7] used pentobarbital, but we have had most unsatisfactory experience with this anaesthetic and now use ether exclusively. Some American workers have had a similar experience. This effect also appears to be related to the strain of animal. Analysis of the adrenals for ascorbic acid can be carried out by any of the common methods. Sayers, Sayers and Woodbury[7] used the Roe - Kuether method[8] but we have used a modified indophenol decolorisation method.[9] In this method the decolorisation of a buffered 2:6-dichlorophenol-indophenol solution is measured photo-electrically exactly 30 seconds after addition of the test solution. It is at least as precise as the Roe - Kuether method and is probably more reliable. It is also much quicker. It is in routine use by Armour Laboratories, who have probably carried out more assays by the Sayers method than any other group. The adrenal ascorbic acid value is expressed in μg per 100 mg of adrenal tissue. Typical results for an assay of a sample of unknown potency against Armour Standard La–1–a are shown in Fig. 2. These data give a log-potency ratio of 0·07, a standard error of the mean of 0·056 and a combined standard deviation of 19·7. At the usual significance level of 1 in 20 this

corresponds to an accuracy of ±16 per cent. for the use of 28 animals. This is well within the limits of precision quoted by Sayers, Sayers and Woodbury.[7] By suitable choice of strain of animal and meticulous experimental technique this precision is always possible, despite reports to the contrary that have appeared in the literature. However, 2 to 3 per cent. of the animals give completely anomalous values, which can easily be discerned. This effect can also be found in the adrenal repair assay. The adrenal ascorbic acid depletion assay is thus about 2000 times as sensitive as the repair assay, while precision and specificity are probably comparable. The former is also very much quicker, but requires more labour and the most meticulous attention to detail. The high sensitivity may in some ways be a drawback, as the extremely dilute test solutions may show the instability characteristic of ACTH in neutral solutions. In routine work we make up our solutions for injection in 0·02 *N* acetic acid, which is tolerated well on intravenous injection and prevents adsorption and other inactivation effects.

Some modifications of the Sayers, Sayers and Woodbury[7] technique have been suggested. Munson, Barry and Koch[10] do not perform the unilateral adrenalectomy before injection but compare the mean total adrenal ascorbic acid in an injected group with the mean of a control group. Sayers, Sayers and Woodbury[7] made a statistical comparison of both methods by using the same experimental data and right-adrenal ascorbic acid values as Munson responses so that identical statistical treatment could be applied to both. They concluded that, to attain the same precision, twice as many animals were required for the Munson method as for the difference method. The Munson method decreases the number of analyses to be performed. It is used in routine work by Armour Laboratories. Stoerk, Porter and Silber[11] have developed a modified Sayers assay in which normal rats are used, the pituitary being blocked by prior administration of adrenal cortical extract. No details are available of this method, but it is understood that this group is now using the usual Sayers assay.

This paper is an attempt to give an account of the chief methods for the assay of ACTH. Only two seem to be of practical value, the adrenal ascorbic acid depletion method and adrenal repair method. The choice between these must depend on the user's special requirements.

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The Ascorbic Acid Depletion Method for the Bio-Assay of Adrenocorticotrophic Hormone, and Preliminary Observations on the Use of Inhibition of Tissue Repair

By BARBARA E. CLAYTON and F. T. G. PRUNTY

(*Presented at the meeting of the Biological Methods Group on Tuesday, October 24th*, 1950)

The adrenal ascorbic acid depletion method for the assay of adrenocorticotrophic hormone has been examined. Certain modifications are given and some results obtained by the authors. For the colony of rats used, λ, the index of precision, had the value $0{\cdot}291 \pm 0{\cdot}044$.

ACTH inhibits the formation of granulation tissue in response to trauma. This fact can be used as the basis of its assay on mice, in which a quantal response is measured.

Sayers, Sayers and Woodbury[1] in 1948 published a method for the assay of adrenocorticotrophic hormone (ACTH) that depended on depletion of ascorbic acid in the adrenals of hypophysectomised rats. During the last six months this method has been used in our laboratory with certain modifications; some results thereby obtained are described below.

Adrenal ascorbic acid depletion

Technique—

Wistar male rats weighing 75 to 130 g were brought into the laboratory at least three days before use; they were fed *ad lib.* on Rowett diet 46* before operation and afterwards on bread and milk. Attempts to maintain their environment at 70° to 75° C were not always successful owing to difficulties with the heating system. When sudden marked fluctuations occurred the technique of hypophysectomy and adrenalectomy were made more difficult, as blood clotting was sometimes impaired. Hypophysectomy was performed under ether anaesthesia. The freshness of the ether was found to be most important if respiratory difficulties were to be avoided. The parapharyngeal approach was used, and, largely by feel, the initial hole in the skull was made with a fine pair of forceps and enlarged with a thick blunt probe, the gland being then removed by suction. A tracheotomy tube was not necessary after some experience had been obtained and is now no longer used. At autopsy the sella was examined for completeness of hypophysectomy.

The left adrenal was removed under ether anaesthesia 18 to 21 hours later. The solution being assayed was injected into the exposed left external iliac vein and one hour later the right adrenal was removed. Excised adrenals were cleaned, weighed to the nearest 0·1 mg on an analytical balance and transferred to trichloro-acetic acid. Ascorbic acid was determined by the dinitrophenylhydrazine method of Roe and Kuethner.[2] New bottles of trichloro-acetic acid should be tested before use, as some samples give a precipitate during the final colour reaction.

Soluble preparations of ACTH were injected in normal saline, less soluble ones were taken up in glacial acetic acid and diluted to 0·02 to 0·01 *N*, as suggested by Morris.[3] The solutions were allowed to stand during the tests in a beaker of water over a tray of ice.

Results

The strain of rat used for assay appears to be most important. The Sprague - Dawley rats in our colony showed great variation in the sizes of adrenal glands, the difference in an individual rat occasionally amounting to as much as 20 mg. In our Wistar rats the adrenal glands usually weighed 7 to 12 mg. In 90 per cent. of the rats the difference in weight between the two adrenals was less than 2 mg and in 23 per cent. the right gland was larger

* Obtained from Heygate and Sons.

than the left. The ascorbic acid concentrations in the adrenals of hypophysectomised but otherwise untreated rats are given in Table I. Some of the percentage differences are much larger than those observed by Sayers *et al.*[1] Where this occurs it is due to the considerably higher concentration of ascorbic acid in the second gland (right); one example of this is given in Table I.

TABLE I

A RANDOM SELECTION OF THE ASCORBIC ACID CONCENTRATION OF THE ADRENALS OF UNTREATED HYPOPHYSECTOMISED WISTAR RATS

Adrenal ascorbic acid, mg per 100 g		Difference in mg (left minus right)	Difference per cent. of left adrenal
Left	Right		
438·2	420·4	+ 17·8	4·0
404·7	402·9	+ 1·8	0·4
365·8	426·6	− 60·8	16·7
494·8	500·0	− 5·2	1·0
367·0	346·0	+ 21·0	5·7
440·0	454·6	− 14·6	3·3
565·0	568·4	− 3·4	0·6
600·0	583·0	+ 17·0	2·8
680·8	642·6	+ 38·2	5·6
644·2	615·2	+ 29·0	4·5
715·0	721·8	− 6·8	0·9
650·0	623·0	+ 27·0	4·1
			Average: 4·1
			Standard Error: ±1·62

An analysis of variance of body weight and initial concentration of ascorbic acid showed no significant effect on the potency of ACTH determined.

The precision of the method is given in Table II. The standard error of the slope is approximately 60, and the standard deviation of 10 slopes is approximately 56, so no evidence of heterogeneity of slopes has yet occurred. The index of precision, λ, is $0{\cdot}291 \pm 0{\cdot}044$, compared with $0{\cdot}176 \pm 0{\cdot}016$ obtained by Sayers *et al.*[1] The method is thus less precise with this strain of rats, and about $2\frac{1}{2}$ to 3 times as many animals are needed by us to give the same degree of precision as these workers got.

TABLE II

PRECISION OF THE ASCORBIC ACID DEPLETION METHOD

Month	Preparation	Number of rats used per preparation	s	b	λ
June	Z3	11	62·7	197·8	0·317
June	200 : 365	13	38·0	160·3	0·237
June	203 : 935	11	40·7	128·7	0·316
July	J10602	12	71·9	263·2	0·273
September	British Organon	10	61·3	272·2	0·225
September	La–1–A	12	57·2	161·4	0·354
October	J20507	12	83·6	262·3	0·318
					Mean = 0·291
					Standard error = ±0·044

Very occasionally it has been found in an otherwise satisfactory assay that one animal failed to respond. Several times, however, when assays have been carried out, it has been found that the responses of the individual animals have shown great variations independent of the dosage of ACTH given; indeed, nearly half may have failed to respond at all. This has occurred both with the older, poorly soluble, preparations and with the newer soluble ones. We have had correspondence with Dr. Hayes and his colleagues of the Armour Company about the results obtained by us on one of these poorly soluble preparations. They noted that the discrepancies we have found were within the limits of variation obtained by them, by both the Sayers method[1] and the Munson modification.[1] They occasionally appear to have similar occurrences in large groups (40 to 50) of animals on any one day, and they ascribe this to biological variation. Nevertheless, for most determinations they reported $\lambda = 0{\cdot}25$. We believe that such aberrant results occur when we periodically use the offspring of certain parents in our colony, and this is being further investigated.

Inhibition of tissue repair

During the treatment of patients with ACTH and cortisone it was noticed by one of us that healing of biopsy wounds was inhibited. This happening independently prompted Howes, Plotz, Meyer and Blunt[3] to study the effect of cortisone on granulation tissue formation in the ears of rabbits. They found that 12·5 mg of cortisone acetate per day per rabbit for 8 to 11 days produced inhibition. They failed to obtain good responses in rats.

It seemed as though the inhibition of healing might be used for the assay of ACTH if smaller laboratory animals, preferably mice, proved suitable. At first some difficulty was experienced in consistently obtaining good granulation tissue in mice. Among methods tried were burning and the injection of turpentine and formalin, but the following method was finally devised.

Technique—

Male albino mice weighing 12 to 18 g were maintained on a diet of Thomson cubes and water. Operation was performed under ether, and an aseptic technique was adopted as far as possible. Fur was removed from the anterior abdominal wall with scissors, a piece of skin about 2 to 3 mm in diameter in the midline of the anterior part of the ventral surface of the abdomen was lifted with fine forceps and clipped off with sharp scissors. The wound was covered with three thicknesses of sterile vaseline gauze, over which was placed the cap of a bottle about 15 mm in diameter. The dressing was held in place with adhesive tape, and the cap prevented any pressure on the wound and any contamination with urine and faeces. Care and attention to detail are essential if consistent controls are to be obtained.

Twenty-eight and a half hours later the mouse was killed, and the dressing was carefully removed. By this technique well-defined granulation tissue was consistently obtained.

The ACTH was given in normal saline as 25 subcutaneous injections, each of 0·1 ml. Injections were given one hour and half an hour before the ulcer was made in the morning, immediately after making it and then hourly, except between 11.30 p.m. and 7.30 a.m. when only two injections were given. With a sufficient dose of ACTH complete inhibition of healing is obtained.

Appearances of the tissues—

Two to three minutes after removal of the dressing, the ulcers were examined about two feet beneath and one foot to the side of an electric light. Figs. 1 and 2 show the appearances of the ulcers. The points to be noted are summarised as follows—

Control healing ulcer	*Inhibited ulcer*
1. Marked hyperaemia.	1. No hyperaemia.
2. Sloping margins.	2. Sharp, clear-cut margins; this appearance is emphasised by the shadow in the third lesion (Fig. 2).
3. Rough, thick floor.	3. Smooth, thin floor.
4. The original vessels in the floor are obscured by new tissue.	4. The original vessels in the floor are still visible.

Histological examination of most ulcers was at first carried out. The ulcer and a large piece of the surrounding skin were removed and placed flat on a thick piece of blotting paper. After 1 minute the skin and paper were immersed in formalin. The paper was removed twenty-four hours later, and the skin was trimmed. The ulcers were sectioned in the transverse diameter and stained with haemotoxylin and eosin. The histological appearances are shown in Figs. 3, 4 and 5. The same differences occur as are seen macroscopically, and it is also found that the thick floor of the control ulcer consists of granulation tissue with marked polymorph infiltration, proliferating fibroblasts and numerous capillaries, while the floor of the inhibited ulcer is very thin and contains only a few polymorphs, some flattened fat cells and no capillaries.

Assay method

In the assays, a preparation of ACTH, Armour 7911, was used as a laboratory standard. Doses of this, ranging between 33 and 62 μg, were given to groups of 10 mice. The percentage

Fig. 1. Left, control healing ulcer; right, inhibited ulcer

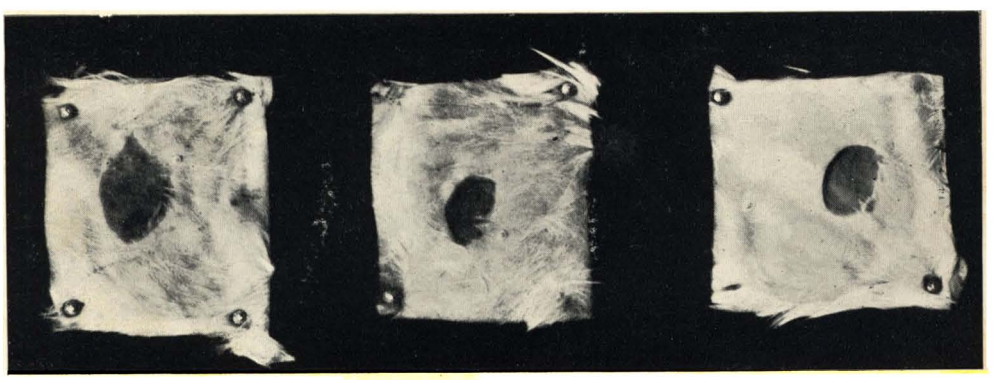

Fig. 2. Left, control healing ulcer; centre, partly inhibited ulcer; right, completely inhibited ulcer

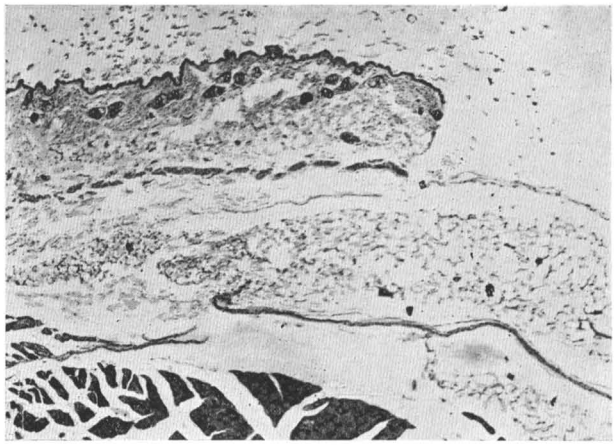

Fig. 3. Section cut immediately after the ulcer was made; stained with H. and E. [× 30

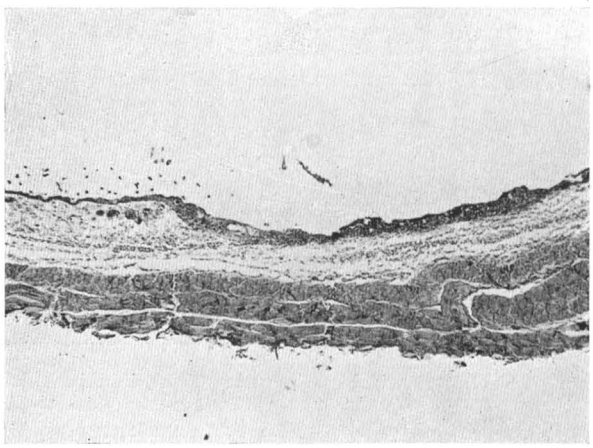

Fig. 4. Section of a healing ulcer; stained H. and E. [× 30

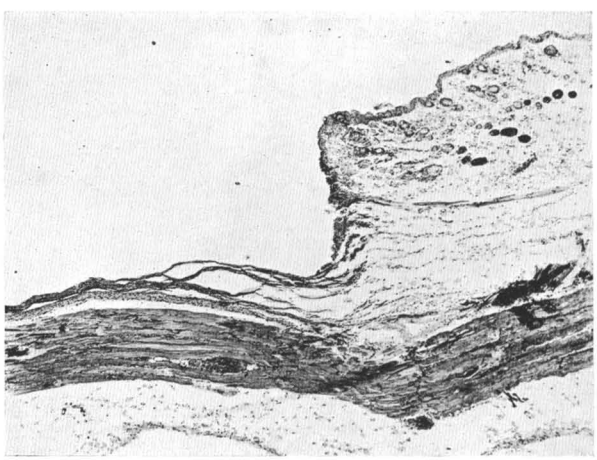

Fig. 5. Section of an inhibited ulcer; stained with H. and E. [× 30

healed in a group was plotted against the logarithm of the dose and gave a sigmoid curve, as shown in Fig. 6. A probit line was fitted, and it was calculated that the approximate 95 per cent. limits of error would be 78 to 128 for 10 animals on each of two preparations, 84 to 119 for 20 animals, and 90 to 112 for 50 animals.

The results of assays on two preparations of ACTH, one from Organon Ltd. and one made by Dr. M. Reiss, are shown in Table III. Thus the potency ratio of Organon ACTH in terms of standard 7911 is estimated at 0·033 with approximate limits of error, 0·071 and

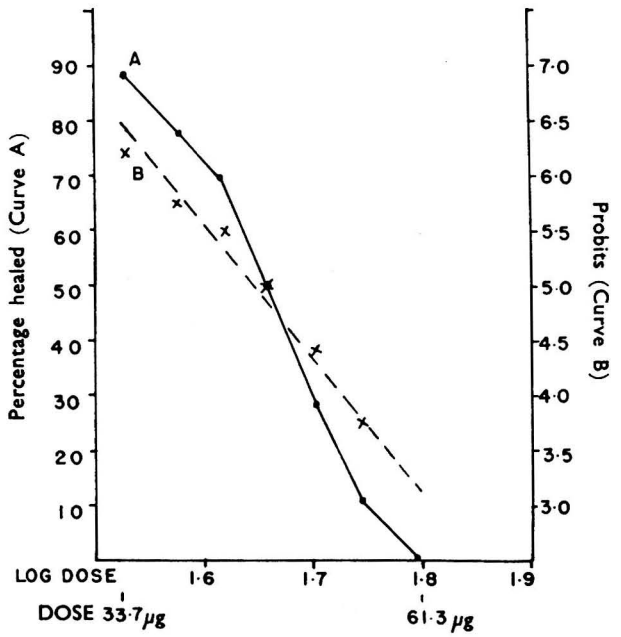

Fig. 6. Dosage - healing curve with adrenocorticotrophic hormone (7911): 7 to 10 mice per group

0·097. The potency ratio of Reiss ACTH in terms of standard 7911 is estimated at 0·265 with approximate 95 per cent. limits at 0·228 and 0·309. This may be compared with estimates by the method of Sayers *et al.*[1]

Owing to shortage of mice these figures were not all obtained on the same day; they are based on the assumption that the dose - response curve did not change in any respect over the period of assay. The test for differences between the three slopes shows no evidence that the slope of the probit line has changed during the period of assay.

These preliminary observations appear to be encouraging. The method is simple and accurate for assaying preparations known to consist of ACTH, and may therefore be of value in controlling its production. The method lacks the specificity of that of Sayers *et al.*,[1] but, in view of its simplicity, may be useful for certain purposes.

TABLE III

RESULTS OF A MOUSE HEALING INHIBITION ASSAY

Organon			Reiss		
Dose in μg	Number of animals	Per cent. healed	Dose in μg	Number of animals	Per cent. healed
418·0	4	75	135·0	4	100
469·0	4	75	151·5	4	50
526·0	4	50	169·4	4	50
590·5	4	50	190·6	4	25

Standard 7911/Organon = 0·083 (Sayer's method 0·070; Limits = 75 to 132 per cent.).
Limits* (P = 0·95) = 0·071 to 0·097.

Standard 7911/Reiss = 0·265 (Sayer's method 0·234; Limits = 46 to 212 per cent.).
Limits* (P = 0·95) = 0·228 to 0·309.

* These limits are approximate 95 per cent. fiducial limits.

ACKNOWLEDGMENTS

We are grateful for statistical assistance given us by Mr. P. Armitage of the M.R.C. Statistics Unit; for technical assistance from Miss J. Hammant in carrying out the assays; from Mr. A. E. Clark in histological work and from Miss J. Dewe in the preparation of the colour drawing. We acknowledge with thanks gifts of ACTH from Dr. J. R. Mote (Armour Company), Dr. W. J. Tindall (Organon) and Dr. Max Reiss (Bristol).

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DISCUSSION

DR. PRUNTY commented that it was important to stress the remarks made by Dr. Overbeck in the discussion on the preceding paper, about the need for repeated injections of ACTH to produce any given response. They had noted in their laboratory that a patient being treated with 40 mg of ACTH daily, divided into four doses of 10 mg intramuscularly at 6-hourly intervals, gave about 1/10th of the biochemical response elicited by the continuous intravenous infusion of 40 mg of ACTH over a 24-hour period. This stresses the importance of continuous administration. In his paper, Dr. Morris stressed the need for hypophysectomy in any ACTH assay. We, too, held the same opinion until we obtained the results we have reported. We do not know of assays based on multiple injections of ACTH at short intervals. One wonders if this technique does not, in fact, produce a "biological hypophysectomy" in the animal, perhaps by direct action on the pituitary. If this were so, it would explain the constant results obtained in their experiments.

Assays of Adrenocortical Hormones on Small Laboratory Animals

BY MARTHE VOGT

(*Presented at the meeting of the Biological Methods Group on Tuesday, October 24th*, 1950)

All biological assays of cortical hormones are carried out on adrenalectomised animals. Mice and young rats are the animals of choice because of the ease and speed with which their adrenals can be removed. Practical details are given for the performance of three such assays.

ALL attempts at assaying cortical hormones either on normal animals or on isolated organs have so far failed; only methods using adrenalectomised animals have met with some measure of success.

In most tests the potency of the assayed material is measured by partial restoration of a functional deficiency resulting from the removal of the adrenals. One test, however, which uses as a measure of activity the fall in circulating eosinophils produced by cortical hormone, is carried out on adrenalectomised animals for the sole purpose of excluding effects due to stimulation of the animal's own adrenals. Since, then, adrenalectomy has to precede any further assay procedure, it is obvious that those species that are easiest to adrenalectomise are also most suitable for these hormone assays. Since the rat is the animal on which mass-adrenalectomy is most conveniently performed, all the tests I shall discuss were originally tried on rats. Soon, however, it was found that, in all but one of these tests, mice show a greater sensitivity to the hormone, not only because of their smaller body weight, but also because of an inherent greater susceptibility. In spite, therefore, of their being somewhat less pleasant to handle and to anaesthetise than rats, all except one of the tests are nowadays done on mice.

There are three biological assays of cortical hormone on rodents of which enough is known to justify their use as routine or screening tests.

(1) The survival test in low environmental temperature, originated in 1938 by Selye and Schenker.[1] ("Cold test.")
(2) The test using deposition of liver glycogen in fasting mice given glucose parenterally, described by Venning, Kazmin and Bell.[2]
(3) The test using the fall of circulating eosinophils in the mouse, devised by Speirs and Meyer.[3]

A method very similar to that of Venning, Kazmin and Bell[2] was published simultaneously by Eggleston, Johnston and Dobriner.[4] Fewer people seem to have used this second method, so that I shall omit its description. There is no reason, however, to suppose that it is less good.

Before describing the different methods, it will be useful to compare their merits or disadvantages—

(*a*) Of the three tests, the "cold test" is easiest to perform, since it requires observation of survival times for a period of an average of 12 to 14 hours, but no further chemical or biological measurements on the adrenalectomised animals.

(*b*) The amount of material required is a little less for the "cold test" than for the liver glycogen test and much less for the eosinophil test.

(*c*) The "cold test" is the only test applicable both to desoxycorticosterone-like compounds and to compounds oxygenated at C_{11}. The former compounds, however, have to be administered in a different way owing to their slow action and do not give a graded response at different dose levels. The other two methods, as far as we know, assay exclusively compounds carrying an oxygen atom in the 11-position.

(*d*) The precision is highest for the glycogen deposition test, lowest for the eosinophil test and appears to be intermediate for the "cold test." The desirable number of animals on each dose or compound is not less than 10 for all tests.

(*e*) Standard and unknown have to be assayed simultaneously in every experiment that uses the "cold test," whereas a standard curve can be constructed for the glycogen deposition test. This means that experiments can be done with unknown solutions alone, and the results read off on a previously prepared standard curve. It is hoped, though it is not certain, that the same will be true for the eosinophil test.

(*f*) The "cold test" gives significant differences between different doses only if the group of rats is made up of litter mates of very nearly the same weight. No such restriction applies to the other two tests.

It may be asked whether there is no method of testing for the influence of cortical substances on electrolyte metabolism. Many such tests have been tried, but none has been found satisfactory. Recently, however, Spencer[5] has developed a method that, though laborious, appears to be very promising. It permits the assay of small quantities of deoxycorticosterone acetate (DOCA) by measuring the rate of sodium excretion after a given load of sodium chloride injected subcutaneously. There has not been enough time since the publication of the method for experience to be gained with it in other laboratories. The method is lengthy and requires precision in all manipulations; it appears to require 0·5 to 4 μg of DOCA per mouse and 12 "selected" adrenalectomised mice per test. The 12 animals are selected from 18 in a preliminary sodium load test carried out without injecting any steroids. On the next day, test, standard and control solutions are given intraperitoneally to 4 mice each and the sodium excretions measured; similar tests are performed on the subsequent two days, changing over the mice used for a particular solution until all mice have in turn received test, standard and control.

A brief description of each test follows, though there is little to add to the published accounts except for the third test.

(1) "Cold test"—

The principle of the test is to accelerate the death of adrenalectomised rats by keeping them at a low environmental temperature. Whereas death at room temperature would occur in 4 to 20 days, the cold environment (in the absence of food) precipitates it, so that it occurs within as many hours. It can be delayed by administration of cortical hormone, and the length of this delay is a measure of the potency of the hormone preparation. The

exposure to low temperature not only shortens the duration of the assay, but also increases its sensitivity and, to a lesser degree, its precision. Age and size of the rat and the litter from which the animal is taken considerably influence its survival time and make careful matching of the groups imperative for obtaining significant differences in survival times with varying doses of hormone (Vogt[6]). The optimal age and size of the animals depends on the particular strain, successful assays requiring vigorous, fast-growing animals. Mice can be used instead of rats, but offer no advantage. Suitable ages and sizes for a particular colony of Wistar rats are, for instance, 21 to 25 days and 37 to 50 g. The variation of weight within a litter should not exceed a few grams, the precision of the results being highest when this variation is smallest. If X samples are tested on X groups of rats in a particular experiment, it is desirable to have 10 litters of X rats each, and to allot one rat of each litter to every group. The effect of variations in weight within a litter is minimised by making the mean weight of all groups equal. It is also advisable to make the sex ratio the same in all groups.

The rats are adrenalectomised on one morning, well fed overnight and used for the assay on the following day. The material to be tested is injected subcutaneously in four doses spaced at intervals of one hour and a half. After the first injection, all animals are simultaneously placed in a cold-room or large refrigerator and only removed for brief periods for further injections or rapid inspection in order to determine the times of death. If doubt is felt about whether a rat is dead or not, it is counted as dead when its corneal reflex has disappeared. The mean survival time of each group measures the potency of each sample. There is a straight line relationship between mean survival time and log-dose of hormone, provided the samples are free from toxic substances. A control group injected with the solvent and two groups treated with different doses of a standard have to be used in every assay in which a quantitative result is desired. If the samples differ in potency by less than 250 per cent. they will rarely produce significant differences in mean survival time. A suitable total dose of extract is 0·1 ml per rat. This may produce an increase of $1\frac{1}{2}$ to 4 hours in mean survival time over the controls, depending among other things on the temperature in the refrigerator. The temperature should be somewhere between $+2°$ and $+7°$ C, according to the vitality of the rats, which will vary with their size and with the strain.

(2) THE GLYCOGEN-DEPOSITION TEST—

The principle of the test is the observation that fasted, adrenalectomised mice only deposit injected glucose as liver glycogen when supplied with cortical steroids carrying an oxygen atom at C_{11}. The test involves liver glycogen estimations on every animal.

Mice weighing from 20 to 25 g are fed on a specified diet, adrenalectomised and kept at a constant temperature. Food is withdrawn on the third post-operative day, and the test is begun on the following morning. Seven hypodermic injections of a mixture of glucose, alcohol and the test substance are given at intervals of 45 to 60 minutes. The mouse is anaesthetised one hour after the last injection. The liver is excised and hydrolysed and its glycogen content is determined. Not less than 6 mice are required for each sample. A glycogen deposition of about 30 mg per 100 g of liver is significant. Since there are seasonal variations in the response, frequent checking with a standard preparation is desirable, but is not required on every occasion as it is in the "cold test." The most accurate results are obtained when between 10 and 40 μg of cortisone are given to each mouse.

(3) THE MOUSE EOSINOPHIL TEST—

The test is based on the fact that injection of cortical steroids oxygenated at C_{11} causes a fall in the circulating eosinophils in the blood of any mammal. The assay is still in the experimental stage, but its sensitivity is such that this fact alone warrants its discussion in this survey. The counting of eosinophils in the blood of the mouse requires some practice, but by using the solution recommended by Speirs and Meier,[3] which dissolves all blood cells except the eosinophils, counting is greatly facilitated. In the description of the details of the test, I shall quote the procedure developed by Dr. Bibile at the Pharmacological Laboratory, Edinburgh (unpublished), but I wish to emphasise that it is still in the process of development.

From 6 to 10 large mice are adrenalectomised, special care being taken to remove all fat surrounding the adrenal along with the gland, in order to avoid regeneration of glandular tissue.

The animals are kept at a steady temperature and supplied with 0·9 per cent. sodium chloride solution instead of drinking water. On the fifth post-operative day, the mouse is warmed until its tail vessels are well dilated, and a drop of blood is obtained from a tail vein for a first eosinophil count. At the same time an injection of 5 μg of adrenaline is given subcutaneously, and a second eosinophil count is made 3 hours later. Those mice responding with a fall in the count are suspect of glandular remnants and are discarded. Immediately after obtaining the second sample of blood, the test substance is injected and its effect on the eosinophil count is observed 4 hours later. A fall indicates adrenocortical activity in a sensitive strain. The test is applicable to doses of 2 μg of cortisone, and possibly to less. The dose - response curve, however, appears to be even flatter than that for the "cold test." It is possible to improve the practicability of the test by using adrenalectomised mice maintained with implants of desoxycorticosterone.

The question of standards has not yet been raised. If we assay a mixture like a cortical extract, it may or may not be justified to use a simple steroid, say cortisone, as a standard. In the "cold test" it is not justified because the dose - response curves are not parallel. In the glycogen deposition test it has been the practice to use Kendall's compound *E* as standard, and this probably introduces little, if any, error, since in that test the potency of an extract is likely to be determined by its content of compounds *E* and the very similar *F*. The same may hold for the eosinophil test, but there is as yet no information on this point.

It is the ardent wish of every worker in the field of biological assays to see the biological test disappear and the assay replaced by a chemical estimation. Until recently, this hope was faint indeed for adrenocortical hormones. To-day, the picture is changing. New chemical micro-methods, particularly chromatography, have also opened new avenues in steroid chemistry. I hope that the task of biological assays will, in the not too distant future, be relegated to its proper field, namely, to help the chemist in the isolation and identification of compounds manufactured by the adrenal gland, and to test synthetic substitutes.

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A Colorimetric Method for Routine Estimation of Calcium in Natural Waters

By F. J. H. MACKERETH

A colorimetric method for determination of calcium in fresh water has been devised. The method is a colorimetric modification of the well known gravimetric technique making use of picrolonic acid, and is suitable for use on small samples (5 ml) of waters containing low concentrations of calcium. The sample should preferably contain 10 to 100 μg of calcium but as little as 1 μg in 2 ml may be estimated. An alternative, less sensitive, but more rapid procedure is also described.

THE following colorimetric method for determination of calcium in fresh water is a modification of the well known gravimetric technique that makes use of picrolonic acid.[1,2,3] It is suitable for small samples of waters containing low concentrations of calcium.

METHOD

The analysis is carried out in Pyrex centrifuge tubes of 10 ml capacity, the tips of which are drawn out to produce a thick-walled capillary base approximately 1·0 cm long and 1 mm bore. The sample containing 10 to 100 μg of calcium (as a rule 5 ml is sufficient in "soft" waters) is pipetted into the tube, and about 3 ml of saturated picrolonic acid solution added. This quantity of reagent represents a considerable excess.

In order to promote crystallisation, which would otherwise be very much delayed by the formation of super-saturated solutions, the sides of the tube are then rubbed down with a stainless steel rod tipped with a little rubber sleeve; the rod is washed into the tube with a drop of distilled water. The tube is then allowed to stand for about 3 hours at room temperature, stoppered with a washed cork to prevent evaporation. Calcium picrolonate is

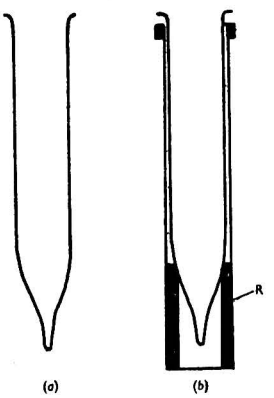

Fig. 1. (*a*) Centrifuge tube. (*b*) Centrifuge tube in cup. The rubber tube, R, inserted in the centrifuge cup supports the tube clear of the base

deposited as a very fine yellow powder, most of which gathers into the capillary tip of the tube. If the concentration of calcium in the sample is comparatively high, *e.g.*, 100 μg per ml, the calcium picrolonate is often precipitated from cold solution as a yellow floc. If this happens, the contents of the tube must be warmed to redissolve the floc and precipitation carried out from the cooling solution; crystallisation will then occur. It is, however, still essential to rub down the sides of the tube when the liquid is cold to ensure complete precipitation. Precipitate that lodges on the side of the tube is loosened with the tipped rod with the usual precautions against loss; the tube is then centrifuged for about 5 minutes at approximately 2000 r.p.m. To protect their capillary tips, the centrifuge tubes are supported in the cups on short lengths of rubber tube. This treatment removes practically all the precipitate into the capillary tip, but any crystals remaining on the sides of the tube must be removed by means of the tipped rod and the tube re-centrifuged. When it is clear that

all the precipitate is in the capillary tip, the excess of reagent and sample is decanted off and the body of the tube washed once with distilled water. This is poured off without disturbing the precipitate. The tube is then half filled with saturated calcium picrolonate solution (at room temperature) and the precipitate gently blown into the body of the tube by means of a capillary jet from a wash bottle filled with saturated calcium picrolonate. The tube is again centrifuged so that the precipitate, now washed once, is returned to the capillary tip of the tube. The wash fluid is now decanted and allowed to drain, as much liquid being removed as possible by touching the meniscus in the capillary with a glass rod, care being taken that the precipitate is not disturbed. The washed calcium picrolonate (results are satisfactory without further washing of the precipitate) is now dissolved in 60 per cent. alcohol by blowing the precipitate into the tube with 5 ml of the alcohol, delivered from a pipette drawn out at the tip so that it may be inserted into the base of the tube. To facilitate solution of the precipitate the tube should be warmed and the contents stirred with the tip of the pipette.

The solution in the tube is now corked to prevent evaporation, and allowed to cool to room temperature. The yellow solution is compared with standards prepared from known amounts of calcium in the same way (a standard containing 100 μg of calcium is usually suitable). For the comparison, an Ogal colorimeter has been used, which, with a 50-mm column, is well suited for dealing with 5-ml samples.

The colour produced bears a linear relation to the calcium content over the range investigated, *i.e.*, 1 to 100 μg of calcium. It is just possible to see the colour produced by 1 μg of calcium as picrolonate dissolved in 5 ml of 60 per cent. alcohol and this may be estimated by comparison with a 10-μg standard prepared in the same way. But comparison becomes difficult with amounts of calcium as small as 1 μg.

The intensity of colour produced by a given quantity of calcium has been found to vary with the composition of the alcohol used for solution and with the temperature.

It is important that the same concentration of alcohol is used for both standard and unknown. A concentration of 60 per cent. has been arbitrarily taken as a compromise between two factors, (1) diminishing colour intensity as the alcohol approaches purity, and (2) diminishing solubility as the alcohol is diluted. The colour variation is not great unless absolute alcohol is used, when a marked decrease in intensity occurs.

As the colour is more intense at higher temperatures it is essential to have the standard and unknown at the same temperature for comparison. Room temperature is satisfactory.

Standards are prepared as described above, or by weighing appropriate amounts of pure calcium picrolonate and dissolving in 60 per cent. alcohol as in usual volumetric technique (35·5 mg of calcium picrolonate dissolved in 100 ml of 60 per cent. alcohol is equivalent to 100 μg of calcium in 5 ml). Since no discrepancy has been observed between standards prepared by the two methods, solubility losses must be very small. This is confirmed by the observation that as little as 1 μg of calcium will produce a precipitate from an original 2 ml of solution to which 2 ml of reagent is added. The standard is stable for several months if protected from evaporation, but on longer keeping the hue alters slightly and the solution assumes a brownish discoloration.

The method is applicable in the presence of 10 times as much magnesium as calcium, but interference is caused by copper, lead, barium and strontium.

Since the completion of this work a previous paper[4] has been brought to the notice of the author in which a substantially similar method of calcium determination is described, but this deals with somewhat larger quantities of calcium (from 500 to 2500 μg).

Results

Known quantities of calcium compared with standard obtained by weighing pure calcium picrolonate and diluting to a concentration of 100 μg of calcium in 5 ml of solution

Calcium taken, μg ..	10	20	30	40	50	60	70
Calcium found, μg ..	10·2	21·0	30·2	40·8	51·0	60·0	70·0
Calcium taken, μg ..	80	90	100	110	120	130	140
Calcium found, μg ..	79·6	89·6	99·7	108·7	119·0	129·3	140·3

Analysis of 1 to 10 μg of calcium compared with 10 μg of standard prepared in the same way as the test samples

Calcium taken, μg ..	1	2	3	4	5	6	7	8	9
Calcium found, μg ..	0·9 to 1·0	1·7	2·8	4·2	4·7	6·0	6·9	7·7	8·8

Eight determinations of calcium in 5 ml of a natural water by the above method gave results varying between 14·2 and 14·8 parts per million.

The following simpler but slightly less sensitive method is suitable for rapid determinations where large numbers of samples have to be analysed. It requires the same equipment and has been studied in this laboratory by Mr. R. J. Holt.

Simplified method

Measure 5 ml of the sample from a pipette into an ordinary 10-ml Pyrex centrifuge tube. One millilitre of saturated picrolonic acid solution is then added from a pipette. The tube is stoppered to keep out dust, heated to 35° C, and then allowed to cool for 3 hours with fairly frequent rubbing down with the rubber-tipped steel rod. If convenient, standing overnight is found to give complete precipitation and is simpler as only one stirring before allowing to settle proved sufficient.

When precipitation of crystalline calcium picrolonate is complete the precipitate is wiped from the sides of the tube and collected at the bottom by centrifuging. The clear solution of excess picrolonic acid is decanted carefully into one cup of the colorimeter and the colour compared with a standard made up by treating 5 ml of water freshly distilled from a tin still in the same manner.

The amount of calcium can be read from a graph, see Table I. As this graph is a straight line, three readings, using standard calcium solutions, are sufficient to define it for a given picrolonic acid reagent.

Table I

Calcium, μg	Colour as per cent. of standard	Calcium, μg	Colour as per cent. of standard
200	36	75	77·8
175	42	50	88·2
150	50·8	25	93·0
125	59·2	10	98·8
100	64·4		
		(standard)	(100)

Interference in this method by other ions will be the same as for the former method. Table II shows the results obtained for calcium in the presence of magnesium.

Table II

Calcium, μg	Magnesium, μg	Colour as per cent. of standard
125	200	52
125	175	49·4
125	150	47·8
125	125	53·2
125	100	52·8
125	75	51·2
125	50	53·4
125	25	51·8
125	0	52·6

Table I shows that the range of this method is from 10 to about 200 μg of calcium in 5-ml samples. It would be possible to raise the upper limit by adding more picrolonic acid reagent but then the lower limit is also raised.

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The Photometric Determination of Small Amounts of Titanium with 8-Hydroxyquinoline

By K. GARDNER

A method is described for the photometric determination of titanium. The procedure uses the colour obtained on extracting solutions of titanium containing hydrogen peroxide, with a 1 per cent. solution of 8-hydroxyquinoline in chloroform. The method is more sensitive than that using the colour given by titanium solutions with hydrogen peroxide only, but the reaction is not very selective. The interference of several cations has been investigated. Small amounts of aluminium, iron or molybdenum can be tolerated, but zirconium and vanadium must be absent. The results of a brief investigation into the composition of the chloroform extract suggest the formation of a "peroxidised" titanium 8-hydroxyquinolinate.

THE principal colorimetric reagents used for the determination of titanium are hydrogen peroxide and phenolic compounds. Hydrogen peroxide has the advantage of good selectivity, while the most useful of the phenolic compounds are probably thymol,[1] chromotropic acid[2] and di-sodium-1:2-dihydroxybenzene-3:5-sulphonate. This last reagent has been fully investigated[3] and is selective and very sensitive.

This paper presents a procedure for the estimation of small amounts of titanium by means of an extraction with an 8-hydroxyquinoline solution.

Previous papers have outlined procedures for the extraction of aluminium,[4,5,6] cerium,[5] gallium[6,9] and indium,[6] molybdenum, iron, tin, copper, nickel and manganese[7] and thallium[9] by means of chloroform solutions of 8-hydroxyquinoline. Gentry and Sherrington use a 1 per cent. solution, which was shown to have wider application than the 0·01 per cent. solution used by Moeller.[8] In the work described below a 1 per cent. solution of 8-hydroxyquinoline in chloroform was used for all extractions.

During experiments using Gentry and Sherrington's method[4] for aluminium, it was confirmed that titanium interfered at the prescribed pH value of 5·0. An attempt to suppress this interference by adding hydrogen peroxide to the aqueous solution was found to result in an extract having a deeper yellow colour. This colour reaction was noted to be much more sensitive than that which hydrogen peroxide alone gives with titanium solutions. Measurements subsequently obtained are given in Table I, and show the drum-difference readings on the Spekker absorptiometer, with H503 and Ilford 601 filters, for various amounts of titanium by the hydrogen peroxide and 8-hydroxyquinoline methods. The final volume, for both methods, was 10 ml. A 1-cm cell with a water - water setting at 1·00 was used. In the hydrogen peroxide method 2 *N* sulphuric acid was used. The relative sensitivities of the two methods are compared in Table I.

TABLE I

RELATIVE SENSITIVITY OF THE HYDROGEN PEROXIDE AND 8-HYDROXYQUINOLINE - HYDROGEN PEROXIDE METHODS. SHOWN AS DRUM-DIFFERENCE READINGS FOR VARIOUS CONCENTRATIONS OF TITANIUM

Concentration of Ti, μg per 10 ml	10	30	50
Hydrogen peroxide method	0·01	0·03	0·05
8-Hydroxyquinoline - H_2O_2 method	0·135	0·420	0·705

The effect of pH value on the extraction of the titanium colour was tested by means of buffer solutions containing 30 μg of titanium in 50 ml of solution. The solutions were extracted with 10-ml portions of 8-hydroxyquinoline solution. Fig. 1 shows that titanium is completely extracted between pH 2·5 and pH 5; the pH measurements being taken immediately after extraction. The effect of pH values above 5 was not investigated because of the instability of hydrogen peroxide in alkaline solutions.

The possibility of using tartrate in order to obtain a colour blank reading was investigated, and Table II gives typical results showing the effect of tartrate concentration. From this table it can be seen that 1 g of Rochelle salt is sufficient to suppress the extraction of titanium

in the absence of hydrogen peroxide, but that in the presence of hydrogen peroxide, 1 g of Rochelle salt only partially suppresses the extraction.

TABLE II

EFFECT OF TARTRATE AND HYDROGEN PEROXIDE ON EXTRACTION OF TITANIUM
All solutions contained 30 μg of titanium

	H_2O_2, 2 ml	Rochelle salt, 0·1 g	Rochelle salt, 1 g	H_2O_2, 2 ml Rochelle salt, 0·1 g	H_2O_2, 2 ml Rochelle salt, 1 g
Drum difference*	0·415	0·085	0·000	0·365	0·135
Percentage extraction	100	5	0	88	33

* The blank value was read from a solution containing 2 ml of H_2O_2 only.

In subsequent experiments a buffer solution of pH 3 was used, for which, in order to carry out a blank reading, it was found necessary to use sodium hydrogen tartrate as the masking agent. The use of this reagent avoids the precipitation of potassium hydrogen tartrate

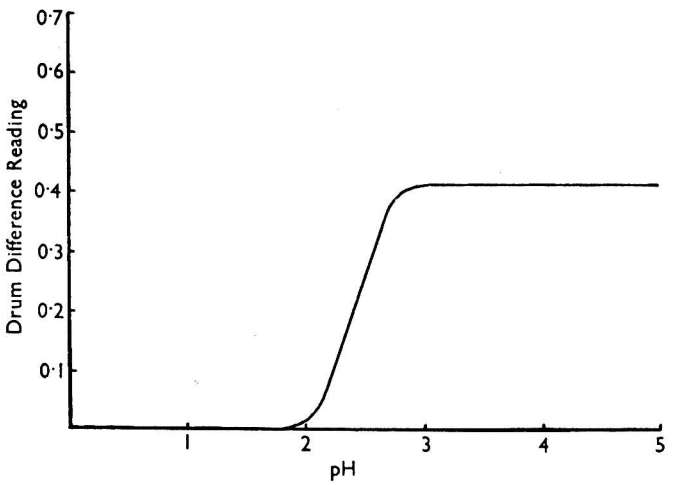

Fig. 1. Effect of pH on extraction of titanium. Tungsten lamp used with 1-cm cell, H503 and Ilfords 601 filter and water - water setting of 1·00 on Spekker absorptiometer

that occurs at pH 3 with Rochelle salt in strong solutions. The final pH of the solution was found to be increased owing to transfer of 8-hydroxyquinoline to the aqueous layer. This increase was of the order 0·1 to 0·2 pH unit (*cf.* reference No. 9).

METHOD

REAGENTS—

All the reagents used conformed to recognised analytical standards.

Titanium solution—Prepare a solution from TiO_2 of more than 99·5 per cent. purity, by fusing the appropriate weight in potassium bisulphate and dissolving the melt in 5 per cent. v/v sulphuric acid. From this solution prepare a dilute titanium solution made up with 5 per cent. v/v sulphuric acid so that 1 ml contains 10 μg of titanium.

8-Hydroxyquinoline solution—A 1 per cent. w/v solution in chloroform.

Sodium acetate solution—A molar solution of salt.

Hydrogen peroxide—A 20-vol. solution.

Sodium hydrogen tartrate—A 65 g per litre solution, adjusted to pH 3·0.

Sodium sulphate—Anhydrous.

The sodium acetate and sodium hydrogen tartrate solutions were purified by extraction with 8-hydroxyquinoline solution. In this way the reagent blank was reduced to a low value.

PROCEDURE—

To the sulphuric acid solution containing titanium in a separating funnel add 2 ml of hydrogen peroxide. Neutralise the solution by dropwise addition of alkali and add 20 ml

of sodium acetate solution. Add immediately sufficient sulphuric acid to give the solution a pH of 3·0, and make up the volume to 50 ml with distilled water. Add exactly 10 ml of 8-hydroxyquinoline solution and shake for 5 minutes. Allow the organic layer to settle and transfer to a dry stoppered flask containing about a gram of sodium sulphate. Measure the absorption of the clear solution on the Spekker absorptiometer using the tungsten lamp with Ilford 601 and H503 filters, a 1-cm cell and a water - water setting of 1·00. The colour blank reading should be obtained by repeating the above procedure with the substitution of 10 ml of sodium hydrogen tartrate solution for 2 ml of hydrogen peroxide. The difference between the two absorptions gives the absorption due to titanium.

The calibration graph was linear over the range 0 to 60 μg of titanium. A sample containing 50 μg of titanium gave a drum-difference reading of 0·70.

The effect of other metal ions

In order to test the interference of certain other cations, appropriate solutions were made up and extractions carried out in the absence and presence of titanium.

Aluminium—

The pH value of 3·0 was chosen for extraction because aluminium is only slightly extracted at this value.[7] Also by using a 601 filter with maximum transmission at 4300 A the effect of aluminium is lessened. Table III gives some typical results showing the effect of aluminium.

Table III

The effect of aluminium on titanium estimation

Aluminium present, μg	600	600	200	100
Titanium present, μg	nil	30	30	30
Drum-difference reading*	0·04	0·03	0·01	0·005

* After deduction of absorption due to titanium.

As tartrate prevents the extraction of aluminium, the quantity of aluminium allowable is 100 μg, which gives an absorption value equivalent to less than 0·5 μg of titanium. By using a spectrophotometer, larger amounts of aluminium could probably be tolerated.

Iron—

Ferric iron is extracted completely at pH 3·0 in the presence or absence of tartrate, and it is therefore included in the blank reading. It is possible to estimate titanium in the presence of 35 μg of ferric iron, but if the titanium content is considerably less than the iron content the blank reading becomes relatively too high for accurate estimation of the titanium.

It was noticed that ferric iron only was extracted at pH 3·0 in the presence of tartrate and that ferrous iron remained in the aqueous layer. This fact might form the basis of a method for the estimation of small amounts of ferric iron in the presence of ferrous iron.

Molybdenum—

Molybdenum interferes because it is extracted at pH 3·0 in the presence of hydrogen peroxide but not in the presence of tartrate. With small amounts of molybdenum a correction graph could be applied or alternatively, it could be extracted at a lower pH value,[7] prior to titanium estimation.

Vanadium—

Vanadium is partially extracted at pH 3·0 in the presence of hydrogen peroxide and completely in the presence of tartrate. It should therefore be absent.

Zirconium—

In the presence of hydrogen peroxide, zirconium is partially extracted and a precipitate appears in the organic phase. No extraction occurs in the presence of tartrate. Zirconium should therefore be absent.

Beryllium, calcium, magnesium and manganese do not interfere.

DISCUSSION OF THE METHOD—

As a spectrophotometer was not available, a rough transmission curve was drawn using the Spekker absorptiometer with different sets of filters; this showed that the extracts obtained with and without hydrogen peroxide had different absorption spectra. The curve given by the extract from the solution containing hydrogen peroxide was displaced towards the blue end of the spectrum.

A chloroform solution of titanium 8-hydroxyquinolinate, $TiO(C_9H_6ON)_2$, obtained by Berg's method,[10] had a much feebler absorption with the 601 filter than that of a solution obtained by extracting an identical amount of titanium in the presence of hydrogen peroxide.

It may be that this compound formed in the presence of hydrogen peroxide is a "peroxidised" titanium 8-hydroxyquinolinate. This possibility seems to merit further study.

The author is indebted to the Director of the Nelson Research Laboratories for permission to publish this work.

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THE ENGLISH ELECTRIC CO., LTD.
NELSON RESEARCH LABORATORIES
STAFFORD

November, 1950

The Determination of Indium in Beryllium Compounds

BY G. W. C. MILNER*

A method is described for the determination of microgram quantities of indium occurring in beryllium compounds. The indium is first separated from the beryllium and certain other constituents of these compounds by an extraction procedure using 8-hydroxyquinoline in chloroform. After concentrating the extracts and decomposing organic matter, the indium is separated from iron and molybdenum by extraction with di-ethyl ether. The concentration of indium is determined polarographically by means of the well-defined step given by it from a base electrolyte consisting of hydrochloric acid, sodium formate and hydrazine hydrochloride.

THE chloro-indic complex ions are reduced from a 0·1 *N* potassium chloride base solution, in the presence of a small amount of gelatin, to give a well-defined and quantitative polarographic step.[1] During investigations on the polarographic determination of indium in refined tin samples[2] this complex was also observed to give a suitable step from a base electrolyte consisting of 2 ml of 50 per cent. v/v hydrochloric acid, 1 ml of sodium formate solution, 68 g in 100 ml of water, 1 ml of 20 per cent. w/v hydrazine hydrochloride solution and 1 ml of 0·2 per cent. starch solution.

The step has a half-wave potential value of approximately −0·6 v. with respect to the mercury-pool anode. This paper describes the application of this reduction step to the determination of indium in beryllium compounds after separating the indium from interfering elements.

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Experimental

Sensitivity—

The sensitivity of the indium step was investigated by adding 25, 50 and 100-μg amounts of indium, dissolved in hydrochloric acid, to three separate 30-ml tall-form beakers and evaporating each solution just to dryness. A 2-ml portion of 50 per cent. v/v hydrochloric acid was next added to each beaker and heat applied to ensure the complete solution of the salts. The 1-ml additions of the sodium formate, hydrazine hydrochloride and starch solutions were then made and the resulting solutions heated just to boiling. After cooling the polarograms were recorded at a temperature of 25° C using a suitable sensitivity on a Tinsley pen-recording polarograph. The sensitivity and quantitative nature of the indium step is shown in Table I.

Table I

Sensitivity and quantitative nature of indium step

Indium concentration, μg	Step height at a sensitivity of 2 microamps.	Step height at a sensitivity of 4 microamps.
25	8·75 divisions	4·5 divisions
50	19·0 "	9·25 "
100	—	18·5 "

Extraction procedure for indium—

Although the reduction of the beryllium ions at the dropping mercury electrode from the above base electrolyte does not interfere with the indium step, beryllium compounds may contain varying amounts of interfering elements; this makes the separation of the indium from as many of these elements as possible almost essential. Elements like iron, copper, bismuth, lead, thallium, tin, etc., reduce at more positive potentials than indium, and cadmium shows a step that coincides with that of indium. Therefore, in applying this step to the determination of indium, cadmium must be completely absent from the final solution; and those elements reducing at more positive potentials must be present in not more than small concentrations, so as not to interfere with the evaluation of the indium step.

Table II

Recovery of indium

Number	Indium added to sample, μg	Indium recovered, μg
1	0	0
2	5	5
3	10	9·5
4	15	15

Indium is quantitatively extracted from aqueous solutions over the pH range 3·5 to 4·5 by a solution of 8-hydroxyquinoline in chloroform[3] and so experiments were carried out to investigate the completeness of this extraction from solutions of beryllium salts adjusted to the correct pH range with the aid of screened methyl orange indicator. Sulphuric acid proved to be a suitable solvent for beryllium oxide and the best solution procedure consisted in dissolving 10 g of sample in 100 ml of 13 *N* sulphuric acid, followed by diluting the resulting solution to 200 ml so as to obviate the danger of crystallisation of beryllium sulphate at room temperature. A 20 per cent. sodium hydroxide solution was used for adjusting the test solutions to the grey change-point of the indicator, slow additions being made in the neighbourhood of the change-point because of the slow re-solution of precipitated beryllium hydroxide.

Details of these experiments were as follows. Dissolve four 10-g portions of a pure beryllium oxide sample in 100-ml portions of 13 *N* sulphuric acid, dilute each to approximately 200 ml and add various amounts of a standard indium solution to give samples containing 0, 5, 10 and 15 micrograms of indium respectively. Add a 20 per cent. sodium hydroxide solution slowly from a burette to adjust each solution to the grey change-point of screened methyl orange indicator and then extract with three separate 15-ml portions of 0·5 per cent. 8-hydroxyquinoline in chloroform. Combine the total extracts of each solution into 50-ml Kjeldahl flasks and remove the chloroform by distillation. Add 4 ml of 20 *N*

sulphuric acid to each flask, destroy the organic matter by the dropwise addition of nitric acid, sp.gr. 1·42, to the fuming sulphuric acid and then completely remove all acids by evaporation. Dissolve the salts in 2-ml portions of 50 per cent. v/v hydrochloric acid, add the 1-ml portions of the other reagents and record the indium polarogram.

The results shown in Table II prove that the indium is completely extracted from the beryllium salts.

Further investigations were conducted to determine the optimum amount of 8-hydroxyquinoline reagent to use and also the minimum number of extractions necessary to give complete separation of the indium. No improvement could be effected in these conditions; so the procedure using three separate 15-ml portions of reagent was adhered to in all subsequent work.

IMPROVEMENT OF SENSITIVITY—

As indium generally occurs in beryllium compounds in very small amounts, it was desirable to apply all possible means for improving the sensitivity. Subsequent investigations showed that it was possible to reduce the volumes of all the reagents constituting the base electrolyte by half, which doubled the sensitivity of the determination. Under these conditions the best defined reduction step was obtained by recording the polarograms with 2 volts applied across the main potentiometer instead of the usual 4 volts.

INTERFERING ELEMENTS—

The 8-hydroxyquinoline extraction procedure restricts the number of possible interfering elements to those that are extracted by it under the conditions of the method. Of these elements, those that have steps with half-wave potential values more negative than −1·0 volt do not interfere with the indium step; the greatest interference comes from those elements that reduce at the dropping mercury electrode at more positive potentials than −1·0 volt. Cadmium gives a step that coincides with the indium step, but fortunately it is not extracted by the oxine reagent. Iron is the most troublesome element, for often 10 g of a beryllium compound contain approximately 1000 μg of iron.

The iron is in the oxidised state after the wet oxidation procedure for removing the organic matter and its interference could be eliminated by chemically reducing it to the ferrous condition, thereby causing the iron step to follow instead of precede the indium step. The acidity of the recommended base electrolyte was found to be too high to permit the complete reduction of the ferric iron by the hydrazine hydrochloride and so the ratio of the 50 per cent. hydrochloric acid to sodium formate was decreased in an effort to improve the reduction. The iron interference proved to be very much smaller in base solutions containing ratios of the above reagents of 1:1, 1:1·5 and 1:2, but unfortunately under these conditions the definition of the indium step deteriorated by assuming an elongated form. These steps were found to be difficult to evaluate and completely unsuitable for this determination. Therefore, the removal of iron from indium by an extraction procedure with *iso*propyl ether or di-ethyl ether was investigated. The results showed that the indium was completely retained in the acid layer free from iron. Moreover, the steps obtained for indium in these layers were well-defined and easy to evaluate.

Molybdenum was observed to give an elongated step in the recommended base electrolyte, the top of which interfered with the indium step. As molybdenum is extracted with indium by 8-hydroxyquinoline, it became necessary to remove the molybdenum completely from the indium. Experiments with *iso*propyl ether and di-ethyl ether showed that although the former reagent does not extract the molybdenum, the latter does so satisfactorily and hence is suitable for the simultaneous extraction of iron and molybdenum from indium. Of the other elements which reduce at more positive potentials than indium, up to 100-μg quantities of copper, bismuth, thallium and tin do not interfere.

The recommended method for the determination of small quantities of indium in beryllium compounds is as follows—

REAGENTS—

Sulphuric acid, 13 *N*—Add 2·5 litres of sulphuric acid, 98 per cent. w/w, to 5 litres of water.

Sulphuric acid, 20 *N*—Add 500 ml of sulphuric acid, 98 per cent. w/w, to 500 ml of water.

Hydrochloric acid, sp.gr. 1·10—Dilute concentrated hydrochloric acid, sp.gr. 1·16, with water, using a hydrometer.

Sodium hydroxide—Twenty per cent. w/v in distilled water.

8-*Hydroxyquinoline*—Dissolve 0·5 g of pure reagent in 100 ml of AnalaR chloroform.

Sodium formate—Dissolve 68 g of pure sodium formate in 100 ml of water.

Hydrazine hydrochloride—Twenty per cent. w/v in water.

Starch solution—Mix 0·2 g of good quality soluble starch with a few ml of water to form a paste and then dissolve in a quantity of boiling water. Cool and dilute the solution to 100 ml.

Screened methyl orange—Dissolve 0·2 g of methyl orange and 0·28 g of xylene cyanol FF in 100 ml of 50 per cent. alcohol.

SAMPLING AND SOLUTION OF BERYLLIUM COMPOUNDS—

(*a*) *Beryllium oxide*—Weigh 10 g of the oxide into a 600-ml lipped conical beaker. Add 100 ml of sulphuric acid, 13 *N*, and evaporate to low bulk. Add a small quantity of water and boil to take most of the oxide into solution. Boil and dilute repeatedly until the diluted solution is clear and free from undissolved particles of oxide. Cool and dilute to approximately 200 ml.

(*b*) *Beryllium sulphate*—Weigh 70·4 g of the sulphate ($BeSO_4.4H_2O$), place in a 600-ml conical beaker and dissolve in 40 ml of sulphuric acid, 13 *N*. Treat onwards as in (*a*) to obtain complete solution. Cool and dilute to approximately 200 ml.

PROCEDURE—

(*a*) *Test solution*—Add 5 drops of screened methyl orange to the sample solution and then sodium hydroxide, 20 per cent., dropwise from a burette. Carry out the sodium hydroxide additions with constant shaking. Just before the indicator change-point is reached, cool and carefully add further alkali until the indicator changes from magenta to grey. Transfer the solution to a 500-ml separating funnel with the minimum amount of distilled water for washing. Add 15 ml of 8-hydroxyquinoline solution, stopper the funnel and shake for 5 minutes. Allow the two phases to separate and run the bottom layer into a dry 50-ml Kjeldahl flask. Wash the aqueous layer left in the separating funnel with 8 ml of chloroform and transfer this also to the Kjeldahl flask. Carry out two further extractions with 15-ml portions of 8-hydroxyquinoline solution as before. Combine the extracts and washings in the Kjeldahl flask and remove the chloroform by distillation over a steam-bath (Note 1).

Add 4 ml of sulphuric acid, 20 *N*, to the Kjeldahl flask and about 0·5 ml of nitric acid, sp.gr. 1·42. Heat gently at first and then strongly to fumes of sulphuric acid. Add further nitric acid dropwise to continue the oxidation of organic matter and finally complete it by the dropwise addition of perchloric acid, sp.gr. 1·54. Cool, dilute with a few ml of water and heat to obtain complete solution of the salts. Transfer the solution to a 30-ml Pyrex tall-form beaker by means of three small washings with distilled water. Evaporate to fumes of sulphuric acid and continue heating to completely remove the acid.

Dissolve the salts in a small quantity of hydrochloric acid, sp.gr. 1·16, and evaporate to dryness. Add 1 ml of hydrochloric acid, sp.gr. 1·10, and warm gently to obtain a clear solution. Transfer to a 25-ml separating funnel using a total of 3 ml of hydrochloric acid, sp.gr. 1·10, for washing. Cool the solution under a water tap, add 5 ml of diethyl ether and shake vigorously for about 30 seconds with cooling under water. Transfer the bottom layer to the original 30-ml beaker, remove the dissolved ether on a steam-bath and finally evaporate to dryness on a hot plate.

Dissolve the salts in 1·0 ml of 50 per cent. hydrochloric acid, by applying very gentle heat, then add 0·5 ml of sodium formate solution, 0·5 ml of hydrazine hydrochloride solution and 0·5 ml of starch solution. Mix well, heat the solution just to boiling-point and cool. Transfer the solution to a polarographic cell, add a small quantity of mercury to form the anode and place in a thermostat for 5 minutes. With 2 volts applied across the main potentiometer and a suitable galvanometer sensitivity setting (Note 2) record the polarogram. Measure the height of the indium step.

(*b*) *Reagent blank solution*—For beryllium oxide samples dissolve 10 g of a spectrographically pure indium-free beryllium oxide sample in 100 ml of sulphuric acid, 13 *N*, and carry out the determination by the above procedure.

For beryllium sulphate samples dissolve 70·4 g of a pure indium-free beryllium sulphate sample in 40 ml of sulphuric acid, 13 *N*, and complete the determination by the above procedure.

Obtain the difference between the step-height reading for the test solution and that for the corresponding reagent blank solution. Deduce the indium concentration by reference to a calibration graph prepared under the following conditions—

Prepare a standard indium solution by dissolving 0·10 g of pure indium metal in 10 ml of hydrochloric acid, sp.gr. 1·16, dilute to 1 litre with distilled water and mix thoroughly. Dilute 10 ml of solution to 200 ml with distilled water so that 1 ml of this solution contains 5 micrograms of indium. Then place 1, 2 and 3-ml aliquots of this solution in 30-ml tall-form beakers and evaporate to dryness. Dissolve the indium salts in 1-ml portions of 50 per cent. hydrochloric acid, warm gently to ensure solution and then add 0·5-ml portions of the sodium formate, hydrazine hydrochloride and starch solutions. Mix thoroughly, heat just to boiling and then cool. Record the polarograms for each solution as described under (*a*) "Test Solution." Measure the step heights and draw the calibration graph by plotting these values against the indium concentration.

Notes

1. It is necessary to commence the removal of the chloroform from the first extraction solution whilst the second extraction procedure is being carried out. If this technique is adopted throughout the extractions and washings there should always be sufficient space in the Kjeldahl flask for all the following extraction and washing solutions.
2. With a Tinsley pen-recording polarograph and a drop-time of 3 seconds, a suitable galvanometer sensitivity was attained when a current of 2 microamperes corresponded to a full-scale deflection of the pen.

The Admiralty has granted permission for this paper to be published.

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Notes

A MODIFICATION OF MILTON'S METHOD FOR ESTIMATION OF TOTAL AVAILABLE CHLORINE IN WATER

R. F. Milton[1] has drawn attention to the fact that all the existing methods for the determination of available chlorine in water, such as the *o*-tolidine, benzidine blue, iodine replacement and modified *p*-aminodimethylaniline methods merely indicate oxidation potential.

He proposed a method for the determination of free chlorine in which the free chlorine reacts directly with cyanide to form cyanogen chloride, which is then allowed to react with pyridine (or a pyridine derivative) to form a quaternary compound that, on subsequent condensation with aromatic amines, produces intensely coloured di-anil derivatives.

This method is stated to be specific for free chlorine (only free bromine reacting similarly), unlike the methods dependent on oxidation-potential mechanisms, which may be affected by iron, nitrite and ammonia, etc.

During a recent investigation of chlorination control of a swimming-bath, use was made of Milton's method (in conjunction with *o*-tolidine and iodine-replacement methods) and certain defects became apparent.

(1) The reagent concentrations were found to be unsuitable.

In the original method, two reagents were employed: (*a*) potassium cyanide, 1 per cent., (*b*) benzidine hydrochloride, 2 per cent., in 25 per cent. pyridine solution. The use of 5 ml of sample was recommended with the addition of 1 ml of potassium cyanide and 5 ml of the pyridine reagent.

It was found, however, that at room temperatures (about 65° F) addition of 5 ml of reagent (*b*) to water caused precipitation of a mass of finely divided crystals of benzidine or the hydrochloride, which was however soluble on the addition of further pyridine, and thus the ratio of pyridine to benzidine hydrochloride, on addition of reagent (*b*) to water, appears to control effective solution. In the present study it was found that a 1 per cent. solution of benzidine hydrochloride in 25 per cent. pyridine was suitable and that it did not cause precipitation when added to the sample.

(2) The method was found to be not specific for free chlorine, but measured total available chlorine concentration, *i.e.*, the concentration of free available chlorine and chlorine combined with nitrogenous compounds in the form of chloramines, etc.

Because of crystallisation on addition of reagent (*b*) to the sample, recourse was made to the modified solution strength described in (1) above. Invariably the figures given by the original method, but using the modified reagents, were comparable with those given by *o*-tolidine and iodine-replacement methods for total available chlorine in water.

Results for swimming-bath water are as shown in Table I, from which it is apparent that the original method measures the total available chlorine concentration and not the free chlorine concentration.

TABLE I

RESULTS OF TESTS ON SWIMMING-BATH WATER

Original method, p.p.m.	Total available chlorine by *o*-tolidine, p.p.m.	Free chlorine (by *o*-tolidine arsenite), p.p.m.
0·30	0·30	0·06
0·63	0·60	0·15
0·94	1·00	0·28

(3) The colours of chlorine solutions of increasing strengths were not of the same tint.

This change in colour was most pronounced; from 0·1 to 1·0 part per million of chlorine the colour changed from yellow to orange to a tan-red. At certain regions of concentration a small difference in concentration of only some 0·03 parts per million gave markedly different colour tints; this makes estimation by comparison with standard chlorine solutions difficult and precludes preparation of satisfactory artificial standards, which are found to be very convenient in methods such as the *o*-tolidine method. Moreover, with the effects mentioned, the solution colours do not lend themselves to convenient measurement by normal photo-electric procedures.

As a result of these observations, further work was undertaken to evolve a method free from these defects, but utilising the same reaction. It was found early in the study that, by adding larger quantities of cyanide and allowing this stage of the reaction to proceed for some 3 minutes before the addition of pyridine reagent, the red colour was completely prevented and yellow-brown solutions obtained. The intensities of colour of these solutions were linear with concentration. The colours produced were easily compared with those from standard chlorine solutions. It was also found that dilute solutions of iodine could be used as satisfactory colour standards.

Separate solutions containing equivalent concentrations of free chlorine and chloramines gave the same colour intensity. Further evidence indicating the sensitivity of the method for the determination of total chlorine in the form of free and combined available chlorine is given in Table II.

TABLE II

SENSITIVITY OF THE METHODS EXAMINED

	Results in parts per million		
	A	B	C
Total available chlorine (by *o*-tolidine and by iodine replacement)	0·19	0·37	0·56
Free chlorine (*o*-tolidine arsenite)	0·15	0·30	0·45
Total available chlorine (method now proposed)	0·19	0·35	0·59

The figures given in Table II for the modification now proposed were obtained by comparing with standard chlorine solutions.

The modified method permits the determination of chlorine down to 0·05 part per million.

Preparation of artificial colour standards can be readily achieved by the use of 0·0005 *N* iodine solution (freshly prepared from 0·1 *N*) and the colours produced are the same as those given by the reaction. It has been found that 2·36 ml of 0·0005 *N* iodine solution gives a colour

equivalent to each 0·10 part per million of chlorine. A series of chlorine solutions were prepared and the total available chlorine was determined by the *o*-tolidine method and by the proposed method against artificial colour standards. The results are shown in Table III.

TABLE III

TOTAL AVAILABLE CHLORINE

	(Parts per million)	
Solution	*o*-Tolidine method	Modified Milton's method
1	0·05	0·06
2	0·08	0·10
3	0·18	0·20
4	0·35	0·33
5	0·44	0·48
6	0·52	0·55
7	0·63	0·60
8	0·77	0·75
9	0·90	0·90
10	1·11	1·08

The figures obtained in this way indicate the true linearity of colour intensity with concentration.

MODIFIED METHOD

REAGENTS—

Potassium cyanide—A 1 per cent. solution.
Benzidine hydrochloride—A 1 per cent. in 25 per cent. pyridine solution.
Iodine solution, 0·0005 *N*—Freshly prepared from standard 0·1 *N* solution.

PROCEDURE—

To 20 ml of sample contained in a 50-ml or 25-ml Nessler tube, add 3 ml of potassium cyanide solution, mix immediately, and allow to stand for 3 minutes.

Add 1 ml of the pyridine - benzidine reagent, mix immediately, allow to stand for a further 3 minutes and compare the colour produced with standards prepared from 0·0005 *N* iodine solution. (2·36 ml of 0·0005 *N* iodine solution is equivalent to 0·10 parts per million of chlorine.) The artificial standards should be made up to a volume of approximately 24 ml, in the same way as the sample, to obtain the best comparison. The standard and sample solutions should be compared by viewing through the length of the Nessler tube.

As an alternative the standard iodine is added to a Nessler tube until the colour produced matches that of the sample in the same volume of solution.

The colour produced by the sample fades slowly on standing.

We are indebted to the Government Analyst, Dunedin, for suggesting this work, and to the Director of the Dominion Laboratory for permission to publish this paper.

REFERENCE

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First submitted *August*, 1950
Amended *February*, 1951

ERRATUM: July (1951) issue, p. 433. The Note by Rudra and Choudhury should bear the date "*August*, 1950."

Apparatus

A MODIFIED APPARATUS FOR STEAM DISTILLATION AND EXTRACTION ON A SEMI-MICRO SCALE

MINOR modifications of an apparatus originally described by the authors[1] enable it to be used for steam distillation without the provision of an external steam generator, and also for extraction on a semi-micro scale.

Steam distillation—To the bottom of the "cold finger" condenser is attached a modified cup A. This has a small orifice in the bottom and is provided with glass hooks that are longer than

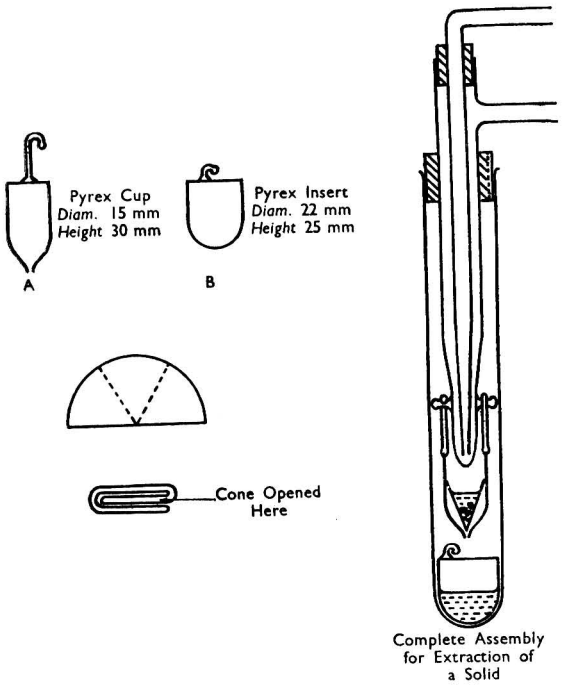

Fig. 1. Details of apparatus as modified for steam distillation and extraction

those in the original design. A small disc of filter-paper, 3·5 cm diameter, is folded to give a cone angle of approximately 40°, see Fig. 1. The paper is inserted into the cup and wetted, a close fit around the upper edge being ensured. About 1 ml of the liquid to be steam distilled is placed in a 25 × 150 mm test tube, together with a few small pieces of porcelain. An equal volume of water is added, the "cold finger" unit attached and the liquid boiled gently. The distillate collects in the filter-paper and can be removed by a capillary pipette. The excess water drips back into the distillation tube and the amount of water admixed with the distillate is therefore kept to an absolute minimum. Some liquids to be steam distilled may already have sufficient water admixed with them. It is only necessary to keep the ratio of water to substance low for those whose solubility is appreciable, *e.g.*, aniline.

If the substance to be steam distilled is a solid, a portion of it may collect on the condenser, whence it may subsequently be removed.

Extraction—The modified cup used for steam distillation may also be used for the extraction of solids. For this purpose the filter-paper cone may again be used or a small Soxhlet type thimble constructed to contain the solid. If it is desired to conserve the extract for crystallisation or weighing, as in a quantitative determination, a small insert *B* is used. This is made so that it is a reasonably close fit inside the distillation tube; a small glass hook attached to the side facilitates its withdrawal.

The solvent is placed in *B*, which is then inserted into the distillation tube. The "cold finger" unit is attached, and the distillation tube is heated in a water or glycerol bath. When extraction

is complete, *B* may be removed if crystallisation of the extract is to be carried out. Alternatively, if the solvent is to be evaporated, as in a quantitative determination, the extraction cup *A* is replaced by one of the normal type and the solvent distilled off.

The modified cup and insert therefore serve to extend the range of the apparatus previously described.

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March, 1951

A HIGH-PRESSURE HYDROGEN SULPHIDE GENERATOR FOR MICROCHEMICAL ANALYSIS

THE customary use of capillary delivery tubes in micro-analysis requires a considerable pressure of hydrogen sulphide to overcome surface-tension effects. Commonly, the necessary pressure is obtained from a Kipp type generator in which the acid head is increased to 50 cm or more.[1,2,3] Alternatively, the gas may be generated at or a little below atmospheric pressure and then pumped through the solution to be treated.[4]

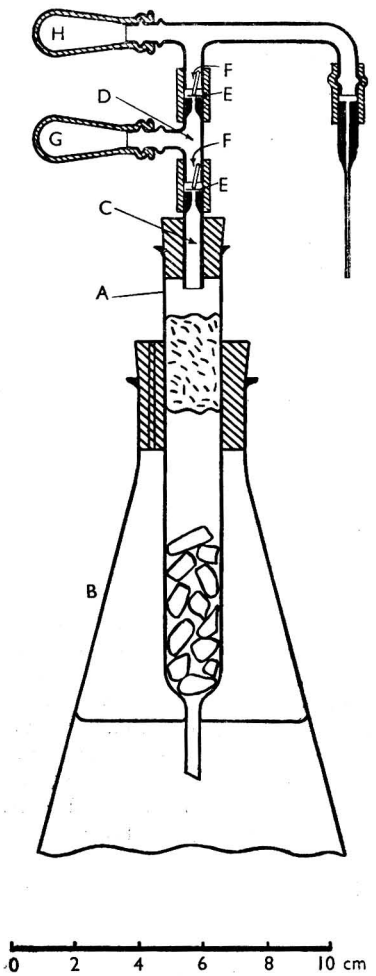

Fig. 1. High-pressure hydrogen sulphide generator

A convenient pump type generator is shown in Fig. 1. By means of a grooved cork, the funnel tube *A* is supported in the mouth of an acid container *B*, a 500-ml conical flask or squat bottle, which is about two-thirds filled with diluted hydrochloric acid (1 + 1) to the level shown in Fig. 1. The funnel tube, which contains broken iron sulphide stick and a glass wool spray-arrestor, is closed by a rubber stopper carrying the pump. The upper ends of portions *C* and *D* are constricted to about 1 mm bore and are ground square and flat. Valves *E E* are discs cut by a cork-borer from thin rubber sheet,[5] and have a maximum lift of about 1·5 mm. Glass studs *F F* lightly press the valves upon their seatings. *G* and *H* are ordinary rubber teats.

The generation of gas does not take place until acid is drawn into the funnel tube by *gently* squeezing and releasing the lower teat *G*. Further operation of this expels air and delivers hydrogen sulphide under pressure high enough to enable a delivery tube with an orifice of about 0·1 mm to be used. This has the following advantages—(1) extremely fine bubbles are produced, so that saturation of the solution takes place with the minimum of gas and without spurting, (2) the rate of delivery is far below the maximum rate of gas-production, so that acid cannot be drawn up into the pump, (3) the reservoir effect of upper teat *H* becomes sufficient to allow continuous bubbling to be maintained by compressing *G* only about twice per minute.

Except for highly precise work, the same delivery tube may be used repeatedly. With the gas still issuing, the contaminated tip is well rinsed and wiped with a scrap of filter-paper. The rate of diffusion of hydrogen sulphide through the fine jet is low, so that, if the generator is in fairly frequent use, gas is available at the first pressure on teat *G*. To avoid possible contamination of the bore of the delivery tube, it is never inserted into the solution to be treated until the gas is passing through it.

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A THERMOSTATICALLY-CONTROLLED HEATING BLOCK FOR SEALED-TUBE REACTIONS

DESIGNED for small-scale organic preparative work, the device shown in Fig. 1 is otherwise useful, *e.g.*, in preparing camphor solutions for molecular weight determination by the Rast method.[1]

The "heating block" proper is a tin can filled with small nails. Pockets for the thermometer and for three or four reaction tubes are old cork-borers, the lower ends of which are burred inwards and plugged with fine steel wool. Reaction tubes are placed in similarly-plugged cork-borers,

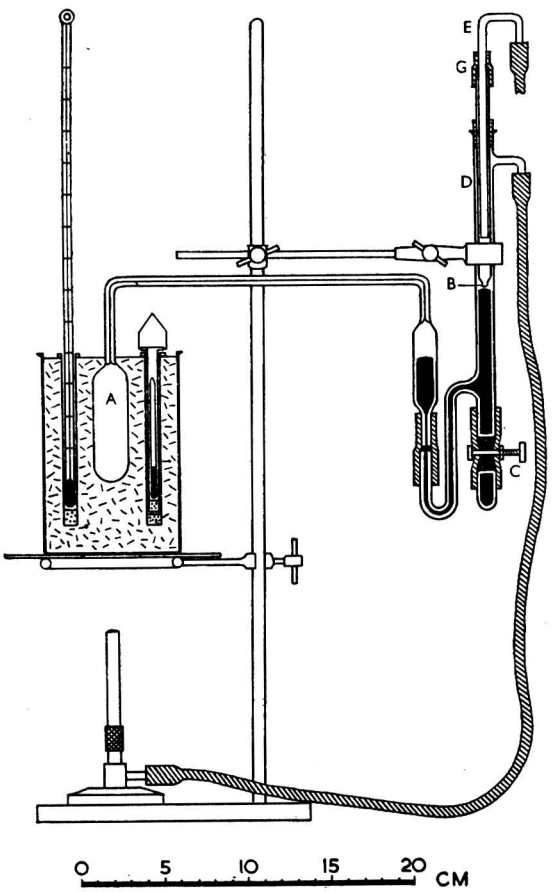

Fig. 1. Thermostatically-controlled heating block

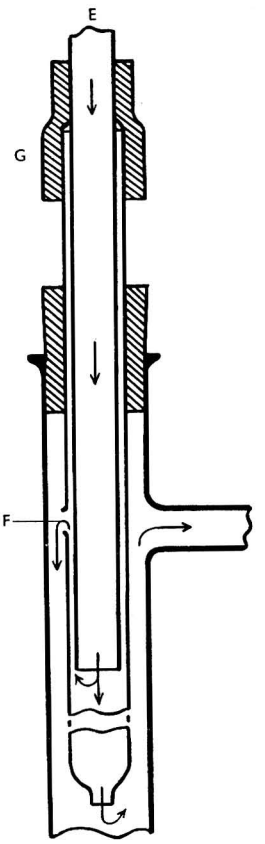

Fig. 2. Detail of by-pass

which slide easily into the pockets and have wire loops for lifting. This permits easy withdrawal of short reaction tubes.

Temperature regulation is effected by the expansion or contraction of air trapped in bulb *A*, which has a capacity of about 20 ml and is submerged in the filling of the "block." Changes in volume are transmitted to a mercury cut-off which controls that portion of the gas supply passing to the burner through jet *B*.[2] The height of mercury in the cut-off, and hence the temperature setting, is adjusted by screw clip *C*.

Satisfactory regulation is obtained by passing only a portion of the gas through the jet, the remainder reaching the burner through variable by-pass *D*. The total gas supply may therefore be adjusted to suit the operating temperature. The by-pass is shown enlarged in Fig. 2. The jet tube, within which gas supply tube *E* is a sliding fit, has a small hole *F* blown in the wall. Gas leaving *E* can reach the burner either through the jet or, after ascending the narrow annulus between the two tubes, through the hole. The length of the annulus, and hence the amount of gas by-passed, may be altered by sliding tube *E* in the soft rubber sleeve *G*. Suitable settings having been found by trial, a paper scale marked every 25° or so may be attached for future reference.

To operate, the by-pass is appropriately set, the burner placed in position and the screw clip slackened. When the thermometer reading is a few degrees below the desired temperature, the screw clip is tightened until the mercury column just closes the jet. After initial fluctuation the temperature should become stabilised, when final adjustments may be made. Normally, any predetermined temperature between 100° and 250° C can be held continuously to within ±2°. Slight re-adjustment may be needed if abnormal change in atmospheric or gas pressure occurs. To avoid possible striking-back, the air-regulating sleeve on the burner may with advantage be replaced by a snugly fitting cylinder of fine copper gauze.

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Ministry of Food

STATUTORY INSTRUMENTS*

1951—No. 1009. The Condensed Milk Order, 1951. Price 3d.

This Order, which came into operation on July 1*st*, 1951, *replaces the Condensed Milk (Control and Maximum Prices) Order*, 1943 (*S.R. & O.*, 1943, *No.* 1396; *Analyst*, 1943, **68**, 332), *as amended by S.R. & O.*, 1945, *No.* 196 *and S.I.*, 1949, *No.* 1028. *The principal change is that maximum prices are prescribed for Special Full Cream Unsweetened Condensed Milk (Evaporated Milk) containing not less than* 10 *per cent. of butter fat. The following definition is given in the Order—*

> "Special full cream" in relation to condensed milk means full cream condensed milk (sweetened or unsweetened) which contains not less than 10 per cent. of butter fat and which is sold in a container bearing a label clearly specifying that the condensed milk in such container contains not less than 10 per cent. of butter fat.

No. 1029. The Meat Products and Canned Meat (Amendment No. 2) Order, 1951. Price 2d.

This Order, which came into operation on June 14*th*, 1951, (1) *restricts the use of milk powder in sausages to "skim milk powder,"* (2) *permits whalemeat to be mixed with other meats provided that the product is labelled and sold as a whalemeat product and* (3) *frees meat paste and fish paste from control.*

It amends the Meat Products and Canned Meat (Control and Maximum Prices) Order, 1948 (*S.I.*, 1948, *No.* 1509; *Analyst*, 1948, **73**, 341), *as amended by S.I.*, 1949, *Nos.* 782, 1303 *and* 2045, *S.I.*, 1950, *No.* 1764 (*Analyst*, 1951, **76**, 119) *and S.I.*, 1951, *No.* 314 (*Analyst*, 1951, **76**, 320), *as follows—*

(*a*) by deleting from Article 1 thereof the definitions of "Fish" and "Fish content";
(*b*) by substituting in Article 1 thereof for the definition "Milk powder" the following definition:—
> '"Milk powder" means skim milk powder.';
(*c*) by deleting from the proviso to the definition of "Meat product" in Article 1 thereof the word "Whalemeat";
(*d*) by adding after the definition of "Tongue" in Article 1 thereof the following definition:—
> '"Whalemeat product" means any product used or prepared for human consumption which is prepared from whalemeat whether with or without another ingredient, but does not include whale oil, whale liver oil, vitamin concentrates or pharmaceutical products.';

* Obtainable from H.M. Stationery Office. Italics indicates changed wording.

(*e*) by adding to the definition of "Excepted product" in Article 1 thereof the item "Meat paste";

(*f*) by substituting in Article 1 thereof for the definition "Meat paste and Fish paste" the following definition:—

"'Meat paste" means any edible paste containing meat and usually known as "meat paste" but does not include any extract usually known as and called "meat extract."';

(*g*) by adding to Article 2 thereof the following paragraph:—

"'(*c*) any whalemeat product clearly labelled and sold as such"';

(*h*) by deleting paragraph (3) of Article 4 thereof (which forbade the use of whalemeat in the preparation of any meat product);

(*i*) by inserting in Article 8 thereof after the words "specified food" the words "whalemeat product or meat paste";

(*j*) by deleting Article 10 thereof (which prescribed the meat and fish content of pastes);

(*k*) by deleting from Part III of the Second Schedule and Part I of the Third Schedule thereof the references to Meat paste;

(*l*) by deleting from Section B of Part I of the Fourth Schedule thereto the reference to Potted meat and Pate de foie.

CURRENT STATUTORY INSTRUMENTS AND STATUTORY RULES AND ORDERS RELATING TO FOOD

This Index of Current Statutory Instruments and Statutory Rules and Orders up to and including March 31st, 1951, includes—

(*a*) *Orders made by or under the authority of the Minister of Food.*

(*b*) *Orders made by the Minister of Food jointly with another Minister or Secretary of State.*

(*c*) *Charges Orders relating to food, made by the Lords Commissioners of His Majesty's Treasury.*

(*d*) *Orders made under the Merchandise Marks Act relating to food.*

(*e*) *Orders and Charges Orders relating to matters which, although not food, are the responsibility of the Minister of Food.*

The Index, Sectional List No. 33, *includes the prices of the individual Orders and may be obtained from H.M. Stationery Office at cost of postage.*

British Standards Institution

NEW SPECIFICATIONS*

B.S. 1715 : 1951. Methods for the Analysis of Soaps. Price 5s.
B.S. 1741 : 1951. Methods for the Chemical Analysis of Liquid Milk. Price 2s.
B.S. 1742 : 1951. Methods for the Chemical Analysis of Condensed Milk. Price 2s. 6d.
B.S. 1743 : 1951. Methods for the Chemical Analysis of Dried Milk. Price 2s.

DRAFT SPECIFICATION

A FEW copies of the following draft specification, issued for comment only, are available to interested members of the Society, and may be obtained on application to the Secretary, Miss D. V. Wilson, 7–8, Idol Lane, London, E.C.3.

Draft Amendment prepared by Technical Committee FCC/4—Solvents and Allied Products.
CN(FCC) 2543—Draft Amendment No. 2 to B.S. 573, Dibutyl Phthalate.

* Obtainable from the British Standards Institution, Sales Department, 24, Victoria Street, London S.W.1.

Book Review

PRACTICAL SPECTROSCOPY. By GEORGE R. HARRISON, Ph.D., Sc.D., RICHARD C. LORD, Ph.D., and JOHN R. LOOFBOUROW, Sc.D. Pp. xiv + 605. London: Blackie & Son Ltd. 1950. Price 35s.

This admirable book was originally published in 1948 in America and is one of the Prentice Hall Physics series, edited by Professor D. H. Menzel. It has now been issued in this country without modification. However, as it deals with the latest developments, it is not likely to become out of date rapidly except where industrial application has been made.

The authors are masters of their subject, and well qualified to write with authority on all branches of spectroscopy. They have wisely included the Raman effect, the Vacuum Ultra-violet and Interferometric Spectroscopy, which have hitherto been regarded as of purely academic interest.

This book is intended to "help the workers in any branch of science to evaluate the aid which the technique of spectroscopy might lend to the solution of his problems." But the exposition is so clear and the advice so experienced that it will doubtless be of assistance to the worker in spectroscopy itself in the solution of his own problems. The book in fact achieves more than is claimed for it. After an introductory chapter, there follow three chapters describing the theory and design of instruments. The relative advantages of prism and diffraction grating instruments are also discussed. A well balanced review of available commercial instruments indicates how these advantages may be utilised. In the chapter dealing with grating spectrographs the classical types of mountings are discussed, including as an illustration the Paschen Runge mounting in the M.I.T. Spectroscopy Laboratory, which is built round a 10-metre concave grating.

Five chapters are concerned with techniques and practical advice in the use of instruments. One of these deals with the testing of optical components, the adjustment of both prism and grating spectrographs and the general care of such apparatus.

In so far as this chapter imparts information that cannot be found either in manufacturers catalogues or in optics textbooks, it provides a useful background for the would-be operator.

Twenty pages are devoted to the illumination of the instrument, including an investigation into the optimum conditions for different types of work. The effects of slit widths on the purity of the spectrum and on the line shape are opposed in spectroscopy. There are normally two requirements, namely, maximum sensitivity for qualitative work, when the arc or spark is focussed on the slit, and uniformity of illumination of the slit, when a lens close to the slit forms an image of the source of the collimating element. In the latter case the exposures are much longer, as the full aperture of the instrument is not employed.

Although photo-electric detectors are finding comparatively small, but increasing, use in emission spectroscopy, photography is still by far the most common method of recording spectra. It is therefore most fitting that a complete chapter is devoted to this part of the procedure. Photographic plates with special emulsions are produced for spectrographic use and great care must be taken in the correct choice of plate and of the best methods for developing and processing. As this treatment is so important, one feels that a little more space might have been given to other methods of processing, such as have been tried in this country (see, for example, "Collected Papers on Metallurgical Analysis by the Spectrograph." London: British Non-Ferrous Metals Research Association. 1945.)

It is doubtful whether the last word will ever be written about light sources in spectroscopy. Many types have been described, including some not mentioned here. What is gained in sensitivity may be lost in stability. It is certain, however, that this field will be of increasing importance when the over-all error of spectroscopic analysis is reduced by replacing the photographic emulsion by a photo-electric cell.

The theory of emission and absorption is dealt with in some detail and it is certain that a full appreciation of these chapters would enable the reader to gauge the suitability of emission or absorption spectroscopy in different wavelength regions to any particular problem.

The exposition of the basic principles of the subject is completed by two further chapters, one dealing with detectors for the measurement of spectral intensities and the other with principles and application of photographic photometry.

Three separate chapters deal with absorption spectrophotometry, qualitative spectrographic analysis and quantitative spectrochemical analysis. Absorption spectrophotometry introduces a new range of terms about which in other works confusion has been caused by the use of different symbols for the same terms. The authors are, however, consistent in this respect and have provided

a clear table defining the terms employed. Readers are warned, however, that they will find different names and symbols for the same terms in some other literature. These terms have been standardised by the list issued in this country (*Analyst*, 1942, **67**, 164). These chapters include methods involving the use of photographic photo-electric and visual instruments, and the authors have not omitted a brief reference to abridged spectrophotometers or absorptiometers.

A general survey of qualitative and quantitative methods of spectrochemical analysis is all that is required, as each worker naturally adapts the technique to his own particular problem. Plenty of useful information is given, however, to enable the usefulness of the method to be deduced.

The subject is completed with chapters on Infra-red, Raman, Vacuum Ultra-violet and High Resolution Spectroscopy. Particular reference is made to the different types of apparatus required and the special techniques used in these fields.

The text throughout is adequately supported by illustrations and specially drawn diagrams, particularly in the earlier chapters, so that the reader is more easily able to follow the later chapters.

The appendix includes wavelength tables showing the sensitive lines of elements arranged first according to elements and secondly according to wavelength. These tables are compiled from the M.I.T. wavelength tables of Harrison.

There is little to criticise in this book, which is notable for its clarity and attention to detail. It is useful, both for reference and as a textbook, by all who either contemplate the employment of spectroscopy or by those already experienced in the use of this important analytical tool.

R. A. C. ISBELL

INTERNATIONAL CONGRESS ON ANALYTICAL CHEMISTRY IN 1952

SOME further details are now available about the arrangements for the International Congress on Analytical Chemistry that is to be held in Great Britain next year.

The Congress will meet at Oxford during the period September 4th to 9th and the scientific sessions will be held in the rooms of the Examination Schools.

Three main Congress lectures by eminent chemists have been arranged. The programme for the scientific sessions is in the hands of a Programme Committee, under the chairmanship of Dr. G. M. Bennett, C.B.E., F.R.S., the Government Chemist, with Mr. L. W. Codd, M.A., of Imperial Chemical Industries Ltd., as Honorary Secretary. This Committee has appointed a number of "Advisers" who are expert in their particular fields, and the draft programme has been divided on a basis of broad techniques.

Papers will be issued in pre-print form before the meeting and the contributors will give only a brief summary of their papers, most of the time being given over to discussion. Arrangements have been made to publish the whole of the proceedings in a special number, or numbers, of *The Analyst*, as soon as possible after the Congress.

During the period of the Congress it is proposed to have in operation working demonstrations illustrating new techniques or special applications of older techniques in analytical chemistry.

In addition to this, and quite separate from it, there will be a trade exhibition comprising apparatus and books. This exhibition will be under the management of Mr. W. Thompson, of Imperial College, London.

Some visits have been planned and, at the week-end, a number of excursions to places of interest will be arranged.

The arrangements at Oxford, for the time being, are in the hands of Dr. F. M. Brewer. Sir Wallace Akers, C.B.E., is Chairman of the Finance Committee; the Honorary Secretary of the Congress is Mr. R. C. Chirnside, F.R.I.C., Research Laboratories, The General Electric Company Ltd., Wembley, England.

Publications Received

LABORATORY DESIGN. Edited by H. S. COLEMAN. Pp. ix + 393. New York: Reinhold Publishing Corporation. London: Chapman & Hall Ltd. 1951. Price $12.00; 96s.

THE B.D.H. GUIDE TO THE ADDENDUM 1951 TO THE B.P. 1948. Pp. 20. London: The British Drug Houses Ltd. 1951.

MEDICINAL CHEMISTRY. Volume 1. By ALFRED BURGER. Pp. xviii + 577. New York and London: Interscience Publishers Inc. 1951. Price $10.00; 80s.

MANURES AND FERTILISERS. MINISTRY OF AGRICULTURE AND FISHERIES BULLETIN No. 36. Ninth Edition. Pp. iv + 96. London: H.M. Stationery Office. 1951. Price 3s.

SILICATE ANALYSIS. By A. W. GROVES, D.Sc., Ph.D., D.I.C., M.I.M.M., F.G.S. Second Edition. Pp. xxiii + 336. London: Thomas Murby & Co. 1951. Price 25s.

TOXIC CHEMICALS IN AGRICULTURE—REPORT OF THE WORKING PARTY. Pp. iii + 16. London: H.M. Stationery Office. 1951. Price 1s.

Report to the Minister of Agriculture and Fisheries of the Working Party on Precautionary Measures against Toxic Chemicals used in Agriculture.

REPORT OF THE INTERDEPARTMENTAL COMMITTEE ON MEAT INSPECTION. Pp. 56. London: H.M. Stationery Office. 1951. Price 1s. 9d.

REPORT OF THE ANALYTICAL METHODS COMMITTEE

THE Report of the Soapless Detergents Sub-Committee, "Examination of Detergent Preparations," reprinted from *The Analyst*, May, 1951, **76**, 279–286, is now available from the Secretary, Miss D. V. Wilson, 7–8, Idol Lane, London, E.C.3; price to members 1s. 6d. and to non-members 2s. 6d.

Papers for Publication in THE ANALYST

THE Editor welcomes Papers and Notes for insertion in *The Analyst*, whether from members of the Society or non-members. They are submitted to the Publication Committee, who decide on their suitability for insertion or otherwise.

A copy of the current Notice to Authors, reprinted from *The Analyst*, 1951, **76**, 385, can be obtained on application to the Editor, *The Analyst*, 7–8, Idol Lane, London, E.C.3. All Papers submitted will be expected to conform to the recommendations there laid down and any that do not may be returned for amendment.

SOUTHERN RHODESIA GOVERNMENT
VACANCY: FOOD TECHNOLOGIST: DEPARTMENT OF HEALTH

APPLICATIONS for the above post are invited from males under the age of 40 years, and holders of the B.Sc. or M.Sc. degree or their equivalent, with experience in food technology. Knowledge and experience of biochemistry, including microbiological assay and of tropical food is also desirable.

Duties will include the investigation of native foods, their storage, preservation and processing, and research with a view to introducing improvements; also the biochemical investigation of clinical material required in connection with nutrition surveys.

Salary Scale: £468 × £66 to £600 × £34 to £668 × £33 to £800 × £100 to £900 × £40 to £1140 per annum, but the commencing salary may be advanced up to four steps in the scale for approved experience subsequent to qualification. The minimum salary applicable to the holder of an M.Sc. degree is £600 per annum. The successful applicant will be stationed in Salisbury and will be required to provide his own accommodation.

Application forms and details of leave conditions, pension scheme, cost of living, marriage and children's allowances, income tax, medical examination, refund of sea and rail fares on appointment, etc., may be obtained from the Secretary to the High Commissioner for Southern Rhodesia, Rhodesia House, 429, Strand, London, W.C.2, to whom completed forms should be returned not later than the 31st August, 1951.

Canvassing will disqualify applicants.

SOUTHERN RHODESIA GOVERNMENT
VACANCY : ANALYTICAL CHEMIST : DEPARTMENT OF GEOLOGICAL SURVEY

APPLICATIONS are invited for the post of Assistant Chemist in the Department of Geological Survey.

Applicants should possess a Specialised Honours Degree of a recognised University, or the A.R.I.C. qualification. Experience in inorganic analysis will be a strong recommendation.

Salary Scale: £468 × £66 to £600 × £34 to £668 × £33 to £800 × £100 to £900 × £40 to £1140 per annum.

In addition to salary a cost of living allowance, at present approximately 20 per cent. of salary, is payable. A successful applicant with an Honours Degree or A.R.I.C. qualification will be appointed to the £600 per annum step in the scale. Additional increments, not exceeding four, may be granted in recognition of previous experience.

The appointment will be subject to the rules and regulations of the Southern Rhodesia Civil Service.

Application forms may be obtained from the Secretary, Office of the High Commissioner for Southern Rhodesia, Rhodesia House, 429, Strand, London, W.C.2, to whom completed forms should be returned not later than the 31st August, 1951.

Details of leave conditions, pension scheme, cost of living, marriage and children's allowances, income tax, medical examination, refund of sea and rail fares on appointment, etc., may be obtained from the High Commissioner's Office.

Canvassing will disqualify applicants.

ROCHE PRODUCTS LIMITED have an opening for assistants in their Analytical Department, age about twenty-five years, of B.Sc. or A.R.I.C. standard, who are trained or wish to do chemical analysis. Write stating qualifications, experience and salary required to the Secretary, Roche Products Limited, Welwyn Garden City, Herts.

THE Research and Development Dept. of the Distillers Co. Ltd. have a vacancy for a chemist to work on biochemical analysis connected with antibiotic fermentation research. Age under 28. Applicants must possess an Honours Degree in Chemistry or equivalent, and experience in biochemical analysis is essential. The vacancy affords good opportunities for any chemist wishing to enter the antibiotic research field, with good prospects of advancement. Salary will depend on qualifications and experience. Non-contributory pension scheme. Apply: Staff Manager, 21, St. James's Square, London, S.W.1.

CHEMIST, qualified, required for control of analytical section of Laboratory at our Shaftmoor Lane Works, Birmingham. Previous experience in this class of work is essential. The position is permanent and pensionable and offers excellent scope for initiative. State age, qualifications and experience to Personnel Manager, Joseph Lucas Ltd., Great King Street, Birmingham.

JOSEPH LUCAS LIMITED require Engineers to fill the following vacancies in the factory which they propose to open in Liverpool for the manufacture of Fuel Injection equipment.

METALLURGICAL ASSISTANTS and experience in at least one of the following:—

1. Production Heat Treatment Checking.
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CHEMICAL LABORATORY ASSISTANTS and experience in one or more of the following:—

1. Process Control (Plating, Blacking, etc.).
2. Analysis of Raw Materials.
3. Analysis of Plating Solutions.

Selected applicants will be expected to spend a short period at the Company's Laboratories in Birmingham prior to taking up their positions in Liverpool. The Company will pay the expenses in connection with such visits.

Application, giving full details of previous experience, should be made in writing to the Personnel Manager, Joseph Lucas (Gas Turbine Equipment) Limited, Shaftmoor Lane, Hall Green, Birmingham, 28.

DUNLOP have two vacancies for assistant chemists at the Research Centre, Birmingham. Applicants should be aged 20—25, with a degree in chemistry. One post is concerned with the development of new analytical methods in the field of rubber and plastics, the other with microchemical analysis. Previous analytical experience, though desirable, is not essential. Salary according to age, qualifications and experience. Applications in writing, quoting ref. A.S 100, To: Personnel Manager, Dunlop Rubber Co. Ltd., Fort Dunlop, Erdington, Birmingham. 24.

MICRO ANALYST desires appointment with an organisation where 18 years' experience and a knowledge of modern methods would be appreciated. Write Box 3780, THE ANALYST, 47, Gresham Street, London, E.C.2.

CHELSEA POLYTECHNIC

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A Course of Lectures and Practical Work on

THE CHEMISTRY AND MICROSCOPY OF FOOD, DRUGS AND WATER

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Lecturer in Charge:

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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, 19th September, 1951, 6-8 p.m., to advise students.

Full particulars can be obtained on application to the Secretary.

Volume 76. No. 905, Pages 445-502 August, 1951

THE ANALYST

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

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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