THE ANALYST

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INTERNATIONAL CONGRESS ON ANALYTICAL CHEMISTRY IN 1952

CONSIDERABLE progress has been made with the arrangements for the International Congress on Analytical Chemistry, which is to be held in Britain in 1952.

It has been decided that the meetings shall be held in Oxford, beginning on 4th September. Accommodation will normally be provided in Colleges, but some hotel accommodation will also be available. The technical sessions will take place in one of the main University buildings.

The period of the Congress will include a week-end, and excursions and visits will be planned to take place during this period.

The arrangements for the Congress are in the hands of a General Committee representing a wide variety of interests and under the Chairmanship of the President of the Royal Society, Sir Robert Robinson, O.M.

The scope of the Congress is under active consideration by an Executive Committee, under the Chairmanship of the President of the Society of Public Analysts and Other Analytical Chemists, Mr. G. Taylor, O.B.E., F.R.I.C., and further details of this and other matters will be published in due course.

It is expected that a meeting of the Board of Section V, Analytical Chemistry, of the International Union of Pure and Applied Chemistry, will be held in Oxford during the same week. Sir Ian Heilbron, F.R.S., is Honorary President, and Professor C. J. van Nieuwenburg, President, of this Section of the International Union.

Sir Wallace Akers, C.B.E., is Honorary Treasurer of the Congress, and the Honorary Secretary is Mr. R. C. Chirnside, F.R.I.C., Research Laboratories of The General Electric Co., Ltd., Wembley, England.

INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY SECTION V—ANALYTICAL CHEMISTRY

THE Section of Analytical Chemistry of the International Union of Pure and Applied Chemistry, organised at the meeting of the Union, September, 1949, has announced its complete membership, which is as follows—

Honorary President—Sir Ian Heilbron, F.R.S.

President—Prof. Dr. C. J. van Nieuwenburg, Technische Hoogeschool, Delft, Netherlands.

Vice-President—Prof. I. M. Kolthoff, University of Minnesota, Minneapolis, Minn.

European Secretary—Prof. Dr. Paul E. Wenger, L'Ecole de Chimie de l'Université, Geneva, Switzerland.

American Secretary—Mr. Samuel E. Q. Ashley, General Electric Company, Pittsfield, Massachusetts.

Other Members—Prof. Dr. Fritz Feigl, Laboratorio da Producao Mineral, Rio de Janeiro, Brazil. Dr. Edward Wichers, National Bureau of Standards, Washington, D.C. Dr. Jan Gillis, University of Ghent, Belgium. Prof. Sir Shanti S. Bhatnagar, Dept. of Scientific Research, New Delhi, India. Mr. R. C. Chirnside, Research Laboratories of The General Electric Company, Ltd., Wembley, England. Prof. F. E. Beamish, University of Toronto, Canada. Mr. N. Strafford, Imperial Chemical Industries Ltd., Manchester, England. Prof. G. Charlot, L'Ecole de Physique et de Chimie, Paris, France.

This membership, which is considered to be temporary, will function until the meeting in New York in September, 1951. At present the Section membership is concerned with the establishment of the two new Commissions on Physico-chemical Constants and on the Expression of Analytical Results. The Commission on New Analytical Reagents and Reactions, which has been functioning for some time, will continue under the sponsorship of this Section.

PROCEEDINGS OF THE SOCIETY OF PUBLIC ANALYSTS AND OTHER ANALYTICAL CHEMISTS

Determination of Fluoride by Etching

By H. AMPHLETT WILLIAMS

(*Read at the meeting of the Society on Wednesday, May 3rd, 1950*)

Synopsis—The reactions involved in the etching of glass have been studied with a view to the development of a specific quantitative method for the determination of small quantities of fluoride. Optimum conditions for high sensitivity have been found by trial, and a method capable of detecting 0·1 μg. of fluoride ion is described. The reactions which take place under these conditions have been found to comprise the dissolution of silica from the glass by the hydrogen fluoride, and the deposition of hydrated silica through hydrolysis of the silicon tetrafluoride formed, with the production of a matt "etching" and the regeneration of hydrogen fluoride. It is concluded that a process in which the fluoride acts as a catalyst provides no sound basis for quantitative analysis.

The etching test, in which the fluoride ion is identified by its property of attacking glass, is the oldest known test for fluorides; it is still one of the most widely used. The reason for its popularity lies in its specificity, for the acids of fluorine are believed to be the only acids that will dissolve glass rapidly and in dilute solutions. Numerous attempts have been made to derive a reliable quantitative method for the determination of fluoride ion by utilising this property, a few with some measure of success. Thirty of the more promising methods published during this century have recently been studied by the author,* but only brief reference can be made here to the literature.

The majority of published methods afford only an approximate estimate of quantity, since they depend upon visual comparison of the depth of an etching produced on a piece of glass that has been exposed to the vapours resulting from treatment of the fluoride with concentrated sulphuric acid with that produced under similar conditions by a known weight of fluoride ion. The minimum quantity of F' which will give a visible etching has been variously reported as between 10 and 0·1 mg., the average being about 0·5 mg.; by using modified techniques higher sensitivities have been claimed by some analysts, notably Woodman and Talbot[1] and Gautier and Clausmann,[2] who found that by heating the reaction mixture to 220° C. in suitable apparatus a few micrograms of F' could be detected. With quantities of that order, however, these workers were unable to obtain consistent etchings.

Several workers have attempted to estimate fluoride ion from the loss of weight observed when a piece of glass is exposed to the hydrogen fluoride evolved by treating the sample, or a purified concentrate obtained from the sample, with sulphuric acid. In Brauns' method,[3] for instance, for the determination of F' in organic compounds, the material is digested in a small Pyrex flask with sulphuric acid and potassium nitrate until decomposition is complete, when the fluorine content is calculated from the loss of weight of the flask. But, unfortunately, almost all minerals containing fluoride ion also contain silica, the presence of which might vitiate an etching test through combining with the fluoride ion as silicon tetrafluoride; and the complete separation of the F' from the Si'''' is a lengthy and unsatisfactory operation. With skilful manipulation, however, Mayrhofer and Wasitzky[4] obtained good results with known weights of sodium fluoride; the average loss in weight of a small, inverted Jena glass funnel, fitted into the top of a crucible in which the purified fluoride residue was heated with concentrated sulphuric acid, being 0·96 mg. of glass per mg. of F'.

In another ingenious method,[2] the fluoride residue after removal of silica is treated with sulphuric acid in a closed platinum crucible containing a small basket of fragments of

* A full review of the literature of etching and other tests for fluoride appears in the Author's Doctorate Thesis entitled "A Study of Existing Methods of Fluorine Analysis and the Development of an Improved Method of General Application" (University of London, 1948), in which is also included the greater part of the experimental work upon which this paper is based.

optical glass (containing 42 per cent. of lead) suspended above the reaction mixture; after treatment for 5 hours at 140° C. the lead fluoride, which is formed on the glass in proportion to the hydrogen fluoride liberated, is dissolved in potassium chlorate solution and the lead determined colorimetrically.

Quantitative etching methods of this type have been used where the quantity of fluoride ion to be determined exceeds 0·1 mg.; where the F' is less than this, they have been found generally unreliable owing to errors inherent in the procedures available for concentration of the F' and its separation from $Si^{\cdots\cdot}$.

The object of the present investigation was to ascertain whether the attractive, if elusive, possibilities suggested by the solvent action of hydrogen fluoride on glass could be utilised as a basis of a specific quantitative method for the determination of very small quantities of fluoride in diverse materials. Although no such object has been attained, it is hoped that the conclusions and the sensitive etching test which follow may be of interest to other analysts seeking the same goal.

Owing to the practical drawbacks of weighing microgram quantities, gravimetric methods were regarded as undesirable and consideration was first given to the principle of producing a permanent etched mark, the extent of which could be determined by optical methods. But before the development of any such method is justified two questions must be answered: whether sufficient sensitivity could be obtained, and whether proportionate and comparable manifestations of the extent of attack on glass could be obtained with known amounts of fluoride. Since the only way in which these questions could be answered was by the method of trial and error, a very large number of etching tests have been performed, under various conditions. The results summarised below show the effect of varying the experimental conditions.

Experimental

The method usually given in the textbooks of analysis was first tried, a known weight of fluoride being heated with sulphuric acid in a platinum crucible covered with a waxed watch glass engraved with a distinctive mark. In the majority of these trials, the fluoride ion was introduced in the form of weighed quantities of intimately ground mixtures of purified calcium fluoride and potassium sulphate, free from silica, containing 1, 0·1 or 0·01 per cent. of fluorine; measured volumes of standard sodium fluoride solution, gently evaporated in the reaction vessel, were substituted occasionally as a check. In the general textbook method the reaction mixture cannot be strongly heated owing to the wax melting, and further trials were carried out with an unwaxed watch glass at a higher temperature. Sensitivity appeared to be slightly higher, as shown in Table I.

Table I

Etchings obtained by "textbook" method

A. *Apparatus*—Platinum crucible with waxed and marked watch glass.

F' introduced, mg.	Time of heating, min.	Etching produced
10	20	deep
1	20	fairly deep
0·5	30	slight
0·3	40	very faint
0·1	40	nil (*bis*)

B. *Apparatus*—Platinum crucible with unwaxed watch glass; higher temperature.

0·5	30	definite but irregular
0·3	30	slight but irregular
0·2	15	faint
0·2	60	faint
0·1	30	nil
0·1	60	nil

Etchings were patchy and the methods insufficiently sensitive, probably owing to the imperfect fitting of the watch glass on the crucible rim and the large area of glass exposed. To ensure better fitting and exactly comparable conditions, a rectangular lead block was cast with two parallel cylindrical cavities. The top of the block was ground flat and the cavities,

after introduction of the fluoride and acid, were covered with a microscope slide held in position by a lead cover over a felt pad. The tests were carried out by heating the lead block in an oil-bath and varying the temperature, time of heating and quantity of acid.

TABLE II

EFFECT OF VARIATION OF TIME AND TEMPERATURE OF HEATING AND QUANTITY OF ACID

Apparatus—Lead block with two cavities, covered with unwaxed microscope slide. First four trials with cavities 7/16 inch in diameter, remainder with new lead block having smaller cavities 5/16 inch in diameter.

F' taken, mg.	H_2SO_4, ml.	Time of heating, min.	Temperature, ° C.	Etching
0·3	2	30	200	very distinct
0·2	2	30	200	definite
0·1	2	30	200	faint
0·05	2	30	200	nil
0·2	1·5	30	200	well defined
0·1	1·5	30	200	slight but definite
0·05	1·5	30	200	very faint (*bis*)
0·02	1·5	30	200	nil
0·05	1·0	30	200	very faint
0·05	2·0	30	200	very faint
0·05	1·5	60	150	very faint
0·03	1·5	60	200	nil
0·03	1·5	90	200	nil

Sensitivity was increased in this way to about 0·05 mg. of fluoride ion, but could not be further increased by variations of time of heating, temperature, volume of acid, nor by

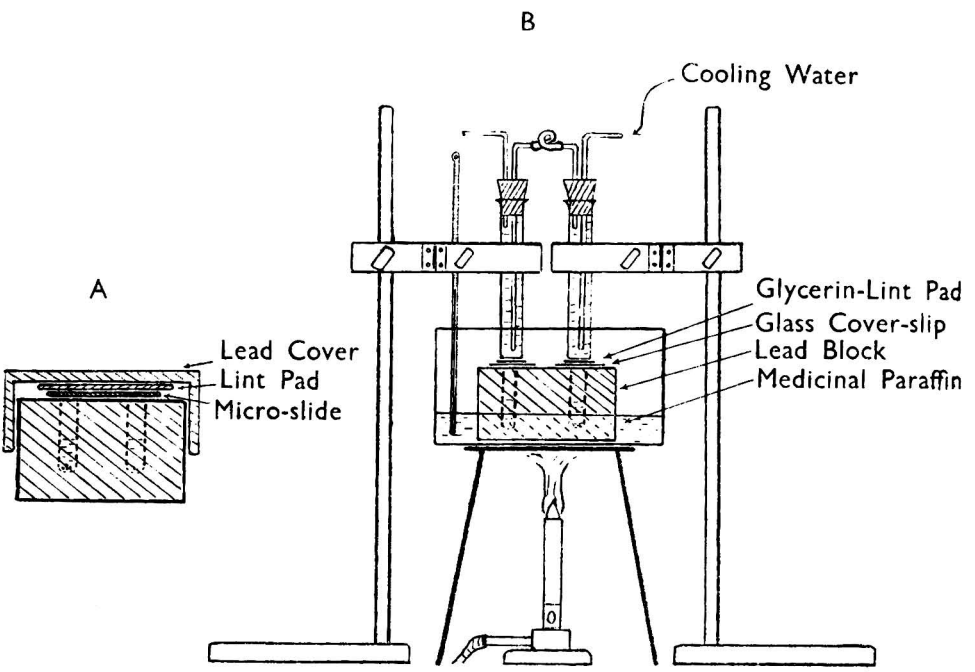

Fig. 1

insertion of lead washers having holes of 1/16, 1/8 and 3/16 inch diameter over the cavities and under the glass slide, which was tried with a view to reducing the area of glass exposed to attack. The temperature could not be raised appreciably above 200° C. owing to the risk of acid creeping up and covering the glass, but 150° C. appeared to be sufficient for the etching.

This is in general agreement with Caley and Ferrer's conclusions,[5] using a similar technique in 1937.

For the next series of trials, on the recommendation of certain of the earlier workers, direct cooling of the glass was introduced. A simple cooling apparatus consisting of flat-bottomed test tubes through which cold water circulated was employed, as shown in Fig. 1; the bottoms of the test tubes resting upon the glass, with pads of lint soaked in glycerin inserted between the glass surfaces to assist conduction. Microscope cover-slips were substituted for the thicker slides, to avoid cracking through the wide difference of temperature on the two sides of the glass.

TABLE III

EFFECT OF COOLING THE GLASS

Apparatus—Lead block; cooled, unwaxed, cover-slips.

F' taken	Time of heating, min.	Temperature, ° C.	Etching
0·2 mg.	30	150	very deep
0·05 mg.	30	150	deep
0·02 mg.	30	150	definite
0·01 mg.	30	150	slight
5 μg.	30	150	nil
5 μg.	60	150	nil
5 μg.	90	150	nil
5 μg.	30	165	faint
5 μg.	30	180	definite
2 μg.	30	180	faint
1 μg.	30	180	nil
1 μg.	45	180	just perceptible
1 μg.	60	180	very faint
1 μg.	90	180	very faint
0·5 μg.	45	180	nil (gave smear when breathed upon)
0·2 μg.	45	180	nil
1 μg.	45	200	2 spoiled, 2 faint but very irregular

The results, given in Table III, show that a remarkable increase in sensitivity can be achieved by keeping the glass cool throughout the test. Since it is difficult to conceive that the rate of attack of hydrogen fluoride on glass would be greater at low temperatures, the increased action is ascribed to concentration of the hydrogen fluoride in the droplets of condensation which form on the glass, and to a prolonged opportunity for attack through avoiding the evaporation from these droplets that probably occurs when the glass becomes heated.

The trials show that by heating for 45 minutes at 180° C. a perceptible etching can be obtained from as little as 1 μg. of fluoride ion, and they suggest that still less might be detectable if the temperature could be raised above 180° C. Difficulties were encountered when this was attempted, the causes being (i) flooding of the cover-slip by creeping or distillation of sulphuric acid, or both, and (ii) deposition of sulphur and water on the cover-slip owing to increased action of the sulphuric acid on the lead. Even at 180° C., etchings were very irregular, the attack producing deep pits in some parts of the glass and a rough, matt surface in other parts, and critical visual comparison of one etching with another was impossible. Moreover, in the course of further trials, a third source of error was encountered, *viz.*, (iii) it was found that after an etching test had been carried out with a relatively large quantity of fluoride ion (20 to 100 μg.), subsequent etchings from very small quantities tended to be erratic and often high, although the cavities were washed out thoroughly between tests. This was ascribed to adsorption of hydrogen fluoride or formation of lead fluoride in the upper part of the cavity, and it was found that distinct etchings sometimes appeared with no added fluoride. Satisfactory "blanks" were only obtained in such circumstances after the lead block had been strongly heated in sulphuric acid, a procedure which caused further corrosion.

These three difficulties render the estimation of microgram quantities of fluoride by etching with lead apparatus impossible, and an alternative material was sought. Eventually a new apparatus was constructed in the following manner.

To contain the reaction mixtures and to provide satisfactory seating for the cover-slips, three platinum tubes, $1\frac{1}{2}$ inches long by $\frac{1}{4}$ inch internal diameter, with flat 1-inch diameter flanges at the open end, were made. The oil-bath, which had proved troublesome owing to the creeping of the fluid (whether medicinal paraffin, wax, or glycerin), was replaced by a solid block of Duralumin, $3 \times 2\frac{1}{2} \times 2\frac{1}{2}$ inches, with three equidistant holes, 5/16 inch in diameter and $1\frac{3}{4}$ inches deep, drilled to take the platinum tubes, and a fourth hole drilled obliquely to the centre of the block to take a thermometer. A few drops of medicinal paraffin, sufficient to cover the bulb of the thermometer, were inserted in the oblique hole, and a small coiled wire spring in each of the other holes; the platinum tubes when inserted fitted snugly into the holes and no conducting liquid was found to be necessary or desirable. Heating was effected by means of an adjustable electric hot plate upon which the block was placed, and cooling by means of a conical flask condenser, the bottom of which was ground flat. The fluoride and acid were placed in the tubes, covered with No. 3, $\frac{7}{8}$-inch circular cover-slips, and the flask, supported by a retort stand, was gently lowered into position. Contact with the cover-slips was effected by the use of glycerin-soaked lint washers, $\frac{5}{8}$ inch in diameter. By means of this apparatus, shown in Fig. 2, three tests may be carried out simultaneously under identical conditions and interference from extraneous reactions and the creeping of oil is obviated.

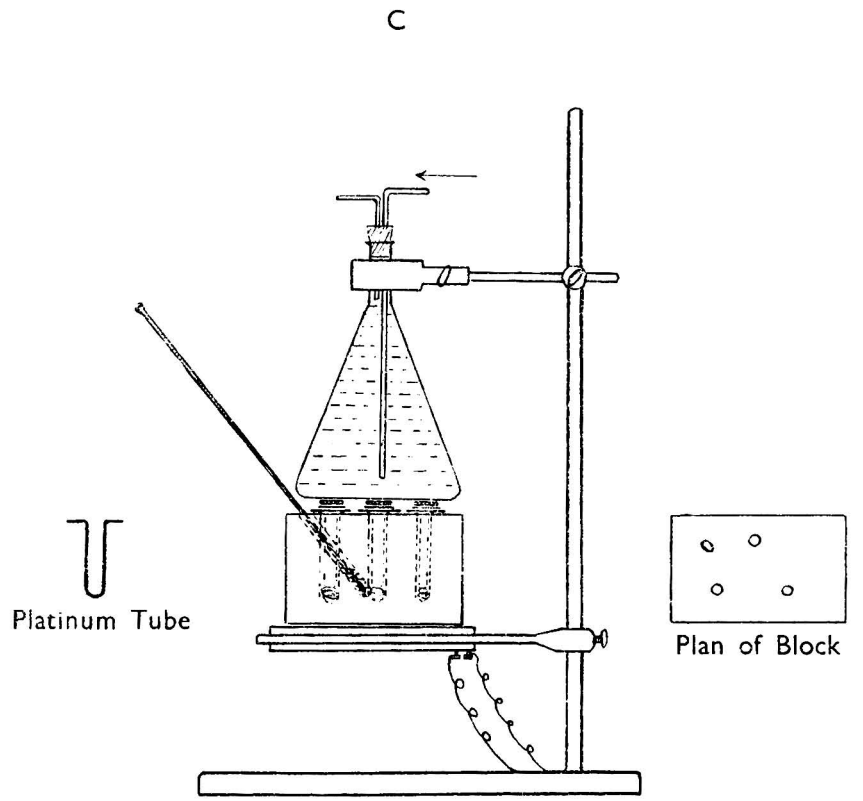

Fig. 2

The most suitable amount of acid, to leave only a small air space but to avoid risk of creeping up to the cover-slip, was found to be 0·7 ml., and this volume of pure A.R. sulphuric acid was used in each tube.

Results with the platinum apparatus proved disappointing and puzzling. The first 30 trials, with quantities of fluoride ion ranging from 0·1 to 10 μg., produced no etchings whatever, no matter what the temperature, time of heating, type of glass or surface condition; even the possibility of lead acting as a catalyst was considered and some lead turnings were added, but without effect.

Eventually another supply of sulphuric acid was tried; this gave a slight etching with 2 μg. of fluoride ion. Four other supplies were then tried, one of which, from the same bottle of ordinary "pure" acid that had been used in the trials with the lead block, gave much better results. Since the principal variant in the different supplies of sulphuric acid was probably the moisture content, it seemed that this might be the essential factor. On determining the strength of various acids by titration and on trying dilutions prepared from the AnalaR acid which had failed to produce any etchings, this proved to be so. The results are shown in Table IV, from which it will be seen that the optimum concentration is apparently 96 to 96·5 per cent. w/w of sulphuric acid (H_2SO_4).

TABLE IV

EFFECT OF CONCENTRATION OF SULPHURIC ACID

Apparatus.—Platinum tubes; cooled cover-slips; heating for 1 hour at 200° C.

F' taken, μg.	Grade of sulphuric acid	H_2SO_4, per cent. w/w	Etching
5	AnalaR (B.D.H.)	97·6	nil
5	"Nitrogen-free" (B.D.H.)	96·8	slight
5	"Technical" (B.D.H.)	92·3	nil
5	"Pure" (T. & M.)	95·8	slight
5	"Pure" (B.D.H.), as used with lead block	96·3	definite
1	AnalaR (B.D.H.), diluted	97·1	very faint
1	ditto	96·6	definite (*ter*)
1	ditto	96·0	definite (*bis*)
1	ditto	95·6	faint
1	ditto	94·4	doubtful
1	ditto	92·2	nil

The trials with different supplies of acid were conducted at 200° C. for 1 hour. At this temperature droplets of moisture condense on the cooled cover-slip and the evolved hydrogen fluoride dissolves in them. The character of these droplets is critical, since if they do not form rapidly enough the hydrogen fluoride may be evolved first and escape under the cover-slip without being dissolved, and if they are too large the solution of hydrofluoric acid formed will be too dilute to attack the glass. According to Olivier,[6] solutions of hydrofluoric acid containing less than 0·00033 per cent. of fluorine do not visibly corrode glass. Moreover, whilst a very fine mist produces an even, matt etching of a type suitable for comparison, larger droplets produce irregular craters in the glass. This seems to be the explanation of the vital importance of the moisture content of the sulphuric acid.

With the exclusive use of 96·5 per cent. sulphuric acid, further trials were carried out with the platinum apparatus to ascertain the optimal conditions, firstly as to temperature and time of heating. Results were not entirely consistent, and even when simultaneous triplicate tests were performed differences in the area or depth of the etchings would occur; but from a large number of trials the following inferences appeared to be justified: (i) that the hydrogen fluoride is not completely evolved at 180° C.; (ii) that a temperature of 220° to 240° C. is necessary; (iii) that the action is not completed by the time the tubes reach 220° or 240° C., a further period at that temperature being necessary; (iv) that no further action occurs at higher temperatures; (v) that gradual heating increases the sensitivity, the best method apparently being to raise the temperature to 120° C. in 45 minutes, to 220° C. in the next 45 minutes, and to maintain at 220° to 230° C. for 45 minutes more; (vi) that no appreciable increase in the sensitivity can be achieved by raising the temperature of the cover-slip during the test; and (vii) that effective contact between the cover-slip and the cooling surface is important. To secure this, the glycerin-soaked lint pad, which tended to enclose air bubbles, was replaced by a small drop (0·02 ml.) of glycerin containing about 2 per cent. of agar, and a fine wisp of cotton wool, placed in the centre of the upper side of the cover-slip.

The type and condition of the glass to be etched was next considered. The experimental requirements of thinness and flatness practically restricted the choice to microscope cover-slips, but careful selection of the grade of cover-slip was found necessary, as some supplies showed a finely pitted surface when examined microscopically with oblique illumination, and variations in sensitivity to attack were also found. Regarding the preparation of the glass surface for etching, it was thought possible that destruction of the molecular symmetry of the surface layer, for example by grinding or by preliminary attack, might render the

substance of the glass more vulnerable. Superior etchings of greater depth and clarity were in fact obtained with the ground and polished glass cover-slips with optically plane surfaces which are specially made for use with haemocytometer cells; the sensitivity, however, was not greatly enhanced. Pre-treatment of cover-slips by immersion in hydrofluoric acid baths of a strength just too low to cause pitting ($\gtrsim$ 1 per cent.) did not appear to render the glass any more susceptible.

Further trials indicated that the state of hydration of the glass surface exerted a marked influence on sensitivity. The best results were obtained when the glass was in a highly hydrated state, and the cover-slips, selected after careful examination from a single packet, were cleaned in chromic acid mixture, soaked in distilled water, and air-dried immediately before use.

On applying the technique and precautions outlined above, a marked increase in sensitivity was obtained with the platinum apparatus, as little as 0·2 μg. of fluoride ion giving a visible etching. The platinum tube overcame the difficulties encountered with the lead block at the higher temperatures, and produced, in the centre of the exposed area of the cover-slip, round matt etchings of a type which would readily admit of comparison, but replicate etchings from similar quantities of fluoride were not always consistent.

Before examining further means of increasing the sensitivity, the question was considered whether the maximum degree of attack theoretically possible had not already been attained. Could it be obtained, a measure of the quantity of silicon separated from the glass in the process of etching or of the amount of combined fluoride ion in the end-products might show whether the whole of the F′ taken had been induced to attack the glass. Attempts to extract and determine the silicon in the deposit of condensed moisture and end-products that formed on the cover-slip during the etching, by solution in alkalies, etc., were unsuccessful owing to high blanks from unetched cover-slips, and determinations of the residual combined F′ by distillation of the deposit on the cover-slip from perchloric acid, followed by thorium nitrate titration, yielded only from one-fifth to one-quarter of the F′ originally taken. It seemed possible, however, that a proportion of both Si^{++++} and F′ might have escaped in the form of silicon tetrafluoride. In these circumstances the only practicable way to ascertain how much glass had been attacked appeared to be by direct weighing of the cover-slip before and after attack. A further series of etching tests, with larger amounts of fluoride, was therefore carried out, the cover-slips being weighed on a microgram balance after standardised cleaning and pre-weighing technique.

TABLE V

LOSS OF WEIGHT OF GLASS COVER-SLIPS

Apparatus—Platinum tubes; cooled cover-slips; heated to 120° C. in 45 minutes, to 220° C. in next 45 minutes, and at 220° to 230° C. for 45 minutes more.

F′ taken, mg.	Weight of cover-slip before etching, mg.	Weight after etching and cleaning, mg.	Loss, mg.	Loss corrected for "blank"	Glass to F′ ratio
nil*	148·535	148·533	0·002		
nil*	145·824	145·819	0·005		
nil*	138·867	138·856	0·011		
nil*	129·086	129·080	0·006		
nil*	139·005	139·001	0·004		
	Mean cleaning blank . .	. . . .	0·006		
1·000	139·900	138·862	1·038	1·032	1·032
1·000	200·760	199·747	1·013	1·007	1·007
1·000	111·808	110·822	0·986	0·980	0·980
0·500	128·804	128·284	0·520	0·514	1·028
0·200	109·432	109·216	0·216	0·210	1·050

* Cover-slip cleaned only.

From the results shown in Table V it can be seen that the average loss of weight by etching was equal to 1·02 mg. per 1 mg. of fluoride ion. This ratio of loss, it may be observed, is higher than any found in the literature (0·5 to 0·96 mg. per 1 mg. of F′). Enquiries elicited the information that the glass used approximated in composition to the "formula,"

$Na_2O.CaO.5SiO_2$; assuming this to be true, the theoretical loss of weight, on the basis of the "equation"—

$$Na_2O.CaO.5SiO_2 + 24HF \rightarrow Na_2SiF_6 + CaSiF_6 + 3SiF_4 + 12H_2O,$$

should be 0·916 mg. per 1 mg. of F′. Even allowing for considerable variation in the composition of the glass, it would seem evident that the attack effected by the F′ had been not less than 100 per cent. efficient.

Since no appreciable increase of sensitivity could therefore be expected, attention was directed to the effect caused by the presence of traces of silica in the fluoride tested, a question

TABLE VI

ETCHING IN THE PRESENCE OF SILICA

Apparatus- Platinum tubes; cooled cover-slips; heated to 120° C. in 45 minutes, to 220° C. in next 45 minutes, and at 220° to 230° C. for 45 minutes more.

F′ taken	Source of F′	SiO_2 added	Source of SiO_2	Etching
1 μg.	CaF_2	2 μg.	Na_2SiO_3 soln.	very strong
1 μg.	CaF_2	4 μg.	ditto	very strong
nil	—	3 μg.	ditto	none
1 μg.	CaF_2	20 μg.	ditto	strong
1 μg.	CaF_2	100 μg.	ditto	weak
1 μg.	NaF	2 μg.	ditto	weak
1 μg.	NaF	4 μg.	ditto	normal
1 μg.	CaF_2	20 μg.	pure SiO_2	very strong
1 μg.	CaF_2	1 mg.	pure SiO_2	normal
1 μg.	CaF_2	1 mg.	purified sand	very strong
nil	—	2 mg.	ditto	none
2 μg.	CaF_2	1 mg.	ditto	strong
5 μg.	CaF_2	1 mg.	ditto	normal
0·4 μg.	CaF_2	10 mg.	ditto	weak
1 mg.	CaF_2	10 mg.	ditto	slightly strong
1 mg.	CaF_2	25 mg.	ditto	normal
1 μg.	CaF_2	1 mg.	powdered glass	strong
0·2 mg.	CaF_2	1 mg. 1 mg. 1 mg.	SiO_2 glass sand	weak
1 μg.	Na_2SiF_6	—	—	normal
0·6 μg.	Na_2SiF_6	—	—	slightly weak
0·3 μg.	Na_2SiF_6	—	—	strong
0·1 μg.	Na_2SiF_6	—	—	just visible
1·5 μg.	Na_2SiF_6	2 mg.	sand	strong
0·7 μg.	Na_2SiF_6	2 mg.	sand	strong
0·4 μg.	Na_2SiF_6	2 mg.	sand	strong
0·2 μg.	Na_2SiF_6	1 mg.	sand	normal
0·1 μg.	Na_2SiF_6	1 mg.	sand	none
0·1 μg.	CaF_2	0·2 μg.	Na_2SiO_3 soln.	strong
0·1 μg.	CaF_2	0·5 μg.	ditto	definite
0·1 μg.	CaF_2	2 μg.	ditto	definite
0·2 μg.	CaF_2	10 mg.	sand	normal
0·05 μg.	CaF_2	10 mg.	sand	none
0·5 μg.	NaF	1 mg.	sand	normal
0·5 μg.	NaF	25 mg.	sand	normal
0·8 μg.	well water	—	—	normal
0·8 μg.	well water, H_2SiF_6 distillate	—	—	normal
0·8 μg.	ditto	10 mg.	sand	normal
0·4 μg.	well water,	—	—	normal
0·3 μg.	ditto	10 mg.	sand	normal
0·26 μg.	ditto	—	—	strong
0·13 μg.	ditto	—	—	strong

of fundamental importance to the general application of an etching process to the determination of fluoride. If sufficient silica is present, the F′ must be evolved, partly at least, in the form of silicon tetrafluoride, and it has been shown by Germann and Booth[7] that pure silicon tetrafluoride does not attack glass, though Rose[8] (in 1850) observed that a slow attack occurred in the possible presence of moisture, and Reich[9] and Olivier[6] also noticed some attack on moistened glass.

To ascertain the degree of separation from silica that would be necessary before microgram quantities of fluoride could be estimated by etching, a number of trials were conducted in which different amounts of silica, in various forms, were introduced into the platinum reaction tubes before adding the sulphuric acid. Representative results are shown in Table VI, the etchings being designated by comparison with the average result produced by a similar weight of F′ without silica.

As the first trials with added silicate recorded in Table VI showed no reduction in the depth or area of the etching, the quantities of silica were increased, ultimately to proportions far in excess of those likely to be encountered in ordinary analytical work. Next, a standard silicofluoride solution was tested, the measured volumes being gently evaporated to dryness in the platinum tubes before addition of sulphuric acid; finally, a sample of a London well water containing 1·3 parts of fluorine per million was tested, with and without additional silica. In many of these tests, some with proportions of Si^{++++} to F′ exceeding 1000 times the theoretical equivalent, there can be little doubt that much, if not all, of the F′ must have been evolved as silicon tetrafluoride; yet, contrary to expectation, definite etchings were obtained. Indeed, although somewhat erratic, the etchings tended to be more marked than those without silica; in a few instances remarkably so and of a rather superior character.

Since silicon tetrafluoride could, presumably, only attack glass after conversion to H_2SiF_6, it seems probable that the larger etchings observed with sub-microgram quantities of fluoride ion when silica was present are connected with the humidity, which, as shown earlier, was found to be necessary for high sensitivity; for silicon tetrafluoride, being more hydrophilic than hydrogen fluoride, as indicated by its vigorous reaction with water, would probably be more quickly and completely absorbed in the moisture condensing on the cover-slip than would hydrogen fluoride, and the H_2SiF_6 formed would then be favourably placed for the production of hydrogen fluoride by dissociation in direct contact with the glass. The proportion of F′ converted to H_2F_2 might thus be from a third to the whole of the F′ absorbed, according to either or both of the reactions —

$$H_2SiF_6 \rightarrow H_2F_2 + SiF_4$$

$$H_2SiF_6 + 4H_2O \rightarrow 3H_2F_2 + Si(OH)_4,$$

the course of which would depend upon local conditions of temperature, moisture, etc. In this connection the importance of cooling the cover-slip, at least in the early stages, may also be explained, for Jacobson[10] has shown that the liberated SiF_4 and H_2O do not react together if the temperature exceeds 30° C., although they immediately combine when cooled to form H_2SiF_6, which subsequently dissociates into H_2F_2 and SiF_4 when the temperature rises.

The possibility of increasing the sensitivity by placing a very small drop of water on the cover-slip before the test, to accelerate and localise the hydrolysis, was suggested by the above hypotheses. It was found, however, that small drops of water, or a thin film, on the underside of the cover-slip quickly disappeared, owing to the desiccating action of the sulphuric acid. A 10 per cent. solution of glycerin was therefore employed instead, a droplet of 0·5 mm. diameter being placed in the centre of the cover-slip. With this solution, the droplet was somewhat reduced in volume at the commencement of the test and did not materially interfere with subsequent comparison of the etchings. The use of the glycerin droplet was found to stimulate attack in its immediate vicinity, 0·2 and 0·1 μg. quantities of fluoride ion giving distinct etchings round the droplet that were several times as great as without the droplet, with or without the addition of 2 mg. of sand, but the sensitivity was not reduced appreciably below 0·1 μg. of F′. This quantity of F′ is probably approaching that required to produce the minimum concentration of free H_2F_2 in the condensed water necessary to attack glass.

The discovery that silicon tetrafluoride produces an attack at least as vigorous as that of hydrogen fluoride under the conditions of the test, however welcome from some points of view, raises questions of fundamental importance to the quantitative application of the etching test. So far as is known to the author, SiF_4 can only attack glass by hydrolysis, thus—

$$3SiF_4 + 4H_2O \rightarrow 2H_2SiF_6 + Si(OH)_4 \quad \ldots\ldots\ldots\ldots \quad \text{(i)}$$

$$\downarrow$$

$$2H_2F_2 + 2SiF_4$$

The residual SiF_4 shown in the equation may be volatilised and gradually lost, or it may continue to undergo hydrolysis until the reaction becomes ultimately—

$$3SiF_4 + 12H_2O \rightarrow 6H_2F_2 + 3Si(OH)_4 \quad .. \quad .. \quad .. \quad .. \quad (ii)$$

The next stage comprises the attack by the liberated H_2F_2 on the glass; this reaction cannot be precisely specified, but the production of more SiF_4 is to be expected, as in the simplified equation usually quoted—

$$2H_2F_2 + SiO_2 \rightarrow SiF_4 + 2H_2O \quad .. \quad .. \quad .. \quad .. \quad .. \quad (iii)$$

Hydrolysis of the secondary quantity of SiF_4 thus formed would lead to a chain reaction, with the F′ assuming the role of catalyst. It cannot be assumed, however, that the liberated H_2F_2 would confine its attack to the glass, and leave the precipitated $Si(OH)_4$ untouched. To some degree, therefore, it may reverse the reaction shown in equation (ii). In any case there can be no prospect of finality. For these reasons any hydrolysis of silicon tetrafluoride would have disastrous consequences to quantitative analysis; consequences, moreover, which could not be avoided by complete elimination of silica from the fluoride residue to be tested.

In view of the evident importance of establishing more clearly what reactions take place during the etching, the end-products remaining on the cover-slip were micro-analysed. For this purpose, 1-mg. etchings were made, with and without added sand, and the moist white deposits found on the exposed areas carefully removed by wiping with fragments of wet filter-paper. Both deposits proved to consist almost entirely of sodium and calcium silicofluorides and water, the weight of the dry silicofluorides in each test being approximately 400 μg. A slight scum was observed on the surface of the sulphuric acid in the platinum tubes after the tests, both with and without sand; this consisted of silicic acid. Except for this small quantity (about 30 μg. of SiO_2), no significant amount of silicic acid or silicate was found in the deposits analysed. But having regard to the loss of weight of the glass, and the loss of fluoride ion unaccounted for, it is evident that a relatively large proportion of dissolved silica must have escaped under the cover-slip in the form of silicon tetrafluoride.

To reduce such losses, further trials were carried out with the cover-slips sealed to the platinum tubes. The under-sides of the cover-slips were moistened with four 0·5-mm. diameter droplets of the glycerin - agar solution, near to the periphery, forming nearly complete seals by capillary attraction when the cover-slips were placed upon the platinum flanges; also the time of heating was increased to $3\frac{1}{2}$ hours, the temperature being very slowly raised to 260° C. during this time.

The resultant etchings exhibited a remarkable form, being apparently raised above the surface of the glass and covered with a powdery micro-crystalline deposit. Repeated cleaning treatment with boiling chromic and sulphuric acid mixture failed to remove this deposit, though silicofluoride would have been dissolved. It was found, however, that much of the deposit could be removed by scraping and that, operating under a dissection microscope with a scalpel, some of the matt surface of the etching could also be removed. On re-examination of some of the previous etchings, obtained from microgram quantities of fluoride ion, it was found that much of the apparent etching could also be removed from these cover-slips in the same way, although this did not apply to the larger quantities employed before the platinum apparatus was devised, where the cover-slips were deeply pitted. Micro-analysis of the substance removed showed that it consisted entirely of hydrated silica. The loss on ignition was 11·3 per cent., suggesting the hemihydrate, $2SiO_2.H_2O$ (13·0 per cent. of H_2O), but the degree of hydration in the silica when deposited may not have been uniform, and may have differed from that existing after the cleaning treatment.

The formation of the matt surfaces on the cover-slip by the deposition of hydrated silica is analogous to commercial matt etching processes, which have been shown to depend upon the growth of fine crystals of silicofluorides or of insoluble double fluorides on the surface of the glass, so protecting the glass underneath from further attack; the pitted appearance is in fact due to minute elevations, the size of which is determined by the degree of saturation of reacting ions and undissociated salts in the etching mixture.[11,12]

The evidence of a deposit of hydrated silica confirms the supposition that hydrolysis of silicon tetrafluoride takes place, and a means of ascertaining the extent of the hydrolysis was sought. Since in the previous experiments the silica remained firmly attached to the glass, it is clear that the loss of weight shown in Table V does not represent the full extent of the attack. Direct determination of the weight of silica deposited could not readily be effected

as the silica could not be quantitatively removed by scraping, and alkali extraction proved unsatisfactory owing to dissolution of the glass, but it was thought that, with the knowledge of the water content of the silicic acid remaining after the cleaning and drying treatment, a sufficiently close approximation might be obtained by finding the loss on ignition of the cover-slips. Corrected for a small "ignition blank," some representative results are shown in Table VII, together with the estimated ratio of glass attacked per 1 mg. of fluoride ion.

With the larger etchings, obtained from 0·5 to 1·0 mg. quantities of fluoride ion, a further, cloud-like, area of attack was noticeable round the periphery of the first sharp etching in trials where the heating was prolonged; this appeared to be due to secondary attack caused by hydrolysis, with further liberation of hydrogen fluoride, of the deposit of silicofluorides first formed; its area extended as the heating was continued.

TABLE VII

TOTAL WEIGHT OF GLASS ATTACKED

Apparatus—Platinum tubes, with liquid-sealed cover-slips; heated gradually to 260° C.

F′ taken, mg.	1·000	0·200	0·100	1·000	0·200	0·100
Silica added	none	none	none	10 mg. glass	1 mg. each sand, glass and silica	10 mg. sand
Duration of test	3½ hr.	2½ hr.	3½ hr.	3½ hr.	2½ hr.	3½ hr.
Loss of weight of cover-slip, mg.	1·001	0·210	0·194	0·118	0·069	0·163
Weight of silicic acid, mg. (further loss on ignition, × 100/88·7)	0·772	0·193	0·174	0·489	0·100	0·162
Total glass attacked, mg.	1·773	0·403	0·368	0·607	0·169	0·325
Glass to F′ ratio	1·8	2·0	3·7	0·6	0·8	3·3

It is noteworthy that the glass attacked per unit weight of fluoride ion was greater with the smaller quantities of F′; this is ascribed to the greater protection afforded by the thicker coating of deposit which forms with larger quantities of F′, and it is probable that with microgram quantities of F′ the ratio of glass attacked would be higher still.

It is now apparent that the losses of weight shown in Table V are understated by the weight of water held by the silicic acid, since the cover-slips were not ignited before being weighed. Moreover, the strange circumstance that, whilst sub-microgram etchings showed inconsistencies of as much as 100 per cent. on visual comparison, the losses of weight from milligram etchings showed fairly close agreement, becomes explicable; the visible etching represents the amount of glass changed into silicic acid, but the loss of weight represents the amount of glass converted into sodium and calcium silicofluorides and silicon tetrafluoride (plus any silicic acid dissolved or dislodged by the cleaning treatment).

In the presence of added silica the losses of weight were extremely variable. If the whole of the fluoride ion were evolved in the form of SiF_4, and the "reaction"—

$$Na_2O.CaO.5SiO_2 + 3SiF_4 + 3H_2O \rightarrow Na_2SiF_6 + CaSiF_6 + 3[2SiO_2.H_2O],$$

proceeded to completion, no appreciable loss of weight would be anticipated. But part of the F′ in the substance under test may be evolved as H_2F_2, although an excess of silica is present, as postulated by Schneider[13]; part of the SiF_4, and part of the deposited silica, may be lost in various ways, and since, moreover, the reactions taking place appear to depend upon fortuitous local concentrations of SiF_4 and H_2O at the glass surface, the variations of weight are not surprising.

To summarise the results of the investigations, an extremely sensitive etching test for fluoride, reacting to 0·1 μg. F′, even in the presence of silica, has been developed; but it has also been shown that the etching of glass, under the conditions necessary for high fluoride sensitivity, comprises a catalytic structural transformation of the silica, which, being capable of proceeding indefinitely, appears to provide no reliable basis for the determination of fluoride.

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DISCUSSION

DR. J. R. NICHOLLS said that this test had always been applied to the etching of glass and it appeared that secondary reactions caused complications. It might, however, be possible to use material other than glass. Suppose the sample were mixed with silica and sulphuric acid and the cover-slip were made of plastic. The liberated silicon tetrafluoride would react with the film of moisture on the cover-slip leaving a deposit of silica and secondary reactions should not take place. The difference in the amount of light passing through the cover-slip before and after the test might be proportional to the deposited silica.

There seemed no reason why a cover-slip of clear fused silica should not be used since that material is very unreactive to hydrofluoric acid.

DR. J. G. A. GRIFFITHS said that, bearing in mind that at least part of fluorine present as hydrofluoric acid or silicon fluoride appears to become fixed on the glass microscope cover-slip as sodium and calcium silicofluorides, and therefore no longer available to "catalyse" the etching process, it would be interesting to know what happens if a silica cover-slip is used in place of a glass slip, and whether the possibility of an apparently interminable cycle of reactions involving hydrofluoric acid would lead to an even smaller quantity of fluorine ion than the quite remarkable minute figure of 0·1 μg. being detected if the glass slip is replaced with a silica slip.

DR. WILLIAMS said, in reply to Dr. Nicholls, that the deposition of silicic acid when silicon tetrafluoride reacted with water provided a very sensitive test for fluorine, and a large number of modifications of this method had been published. In the quantitative variations the silicic acid formed was usually determined colorimetrically by development of the molybdenum blue complex, but he did not know of any procedure by which microgram quantities or less of fluorine could be estimated.

Dr. Griffiths' suggestion was an interesting one, but he did not think the sensitivity could be increased much further because a certain minimum strength of hydrofluoric acid, said by Olivier to be 0·0003 per cent., was necessary for the solution to attack glass at all.

Observations on the Spectrophotometric Estimation of Vitamin D

BY H. E. COX

(*Read at the meeting of the Society on Wednesday, May 3rd*, 1950)

SYNOPSIS—The International Unit of vitamin D has recently been changed by the World Health Organisation, but the new unit has absorption constants similar to those of the old unit and gives a similar reaction with the acetyl chloride - antimony chloride reagent first proposed by Nield, Russell and Zimmerli. A study of this method shows that it works well for control purposes provided that (*a*) the amount of vitamin D present is sufficient, (*b*) the amount of vitamin A is not more than about five times that of the vitamin D and (*c*) suitable correction is made for the absorption due to certain other sterols. Data are given on interfering substances and on commonly occurring fats that are used in the preparation of commercial vitamin products. When there is too high a concentration of vitamin A it may be separated by chromatography.

THERE is at present no recognised chemical, physico-chemical or even microbiological method applicable to the estimation of vitamin D in foodstuffs. The biological method is so costly and time-consuming that there is great need for a simpler or cheaper process and every contribution to this end is to be welcomed. To appreciate the problem it is necessary first to define what has to be determined and then to consider possible methods and probable

interfering substances. Until quite recently the International Unit of vitamin D was defined in terms of a solution of irradiated ergosterol that was in fact mainly a solution of vitamin D_2 and was equivalent to 0·025 μg. of calciferol. Calciferol is now available as a synthetic chemical of a high degree of purity, has the empirical formula $C_{28}H_{43}OH$, and is the subject of a specification in the British Pharmacopoeia. More recently the World Health Organisation, which is the body charged with the duty of defining units and issuing appropriate international standard preparations of certain of the vitamins, has decided that the solution of irradiated ergosterol, being mainly of vitamin D_2, has not proved to be sufficiently representative of the D vitamins. Also a suggestion made at an earlier conference that the standard should be replaced by pure crystalline calciferol is now unacceptable. So the standard has been re-defined in terms of the alcohol $C_{27}H_{43}OH$, formerly known as vitamin D_3. This compound differs from calciferol in the nature of its side-chain; the methyl group at C_{24} is absent and there is one double bond less. It is available as a pure substance in crystalline form and has a melting-point of 87° to 89° C., $[\alpha]_D^{20}$ + 110° and $E_{1\,cm.}^{1\%}$ at 265 mμ. = 490; 0·025 μg. of this substance now becomes the International Unit of vitamin D.

It will be noted that this substance is defined as having a high extinction coefficient; in 1 per cent. solution in alcohol it is as high as 490, and ϵ, the molecular extinction coefficient, is 18,800. So it is evident that it would be quite easy to determine the amount of this substance in a pure solution or in the absence of interfering substances.

Besides natural products containing vitamin D, there are many proprietary preparations that contain and claim it, and the Labelling of Food Order requires that certain specified proportions of the normal daily requirement (500 I.U. of vitamin D) shall be present. This brings an insistent demand for its estimation, and it might be said that it is the duty of the Public Analyst to determine it. In general, he cannot do so. The vitamin is always associated with the fatty portion of the food in which, of course, it is soluble. Even if the fat is extracted, and saponified and the unsaponifiable matter dissolved in pure solvent, such as *cyclo*hexane, there is almost always enough impurity to mask the specific absorption curve of the vitamin. Nearly all the sterols associated with the unsaponifiable portion of fatty oils have absorptions in the region of 260 mμ., so that a direct method such as is used for vitamin A is inapplicable. Moreover, if vitamin A is also present in quantity substantially greater than vitamin D, its absorption will overlap the 260 mμ. region.

The well known Carr - Price reaction for vitamin A was, of course, very attractive and as long ago as 1936 it was observed that vitamin D or calciferol gave a pink or orange colour with the same reagent, but it was not sufficiently stable and reproducible for a quantitative method, and many substances interfere. In 1940, however, Nield, Russell and Zimmerli[1,2] introduced the use of acetyl chloride into the reagent and since then various improvements have been made or proposed. The reagent is a solution containing 20 per cent. w/v of antimony chloride to which is added 4 per cent. of pure acetyl chloride. The reagent must be fresh and must be quite free from alcohol. When 9 volumes of this reagent are added to 1 volume of a solution of vitamin D in *cyclo*hexane or in chloroform, an orange colour is rapidly developed; it reaches a maximum in about 4 minutes and retains it for about 12 minutes. The optimum conditions of time and temperature have been studied by various authors. The colour is fairly specific and has a characteristic absorption curve with a maximum at 500 mμ. At this wavelength the value of $E_{1\,cm.}^{1\%}$ is 1880, a figure that makes the test four times as sensitive as the absorption at 265 mμ.

Vitamin A with this reagent gives the well known blue colour; this fades quickly and is, indeed, somewhat suppressed by the acetyl chloride. The blue colour has $E_{max.}$ at about 620 mμ., which is well clear of the 500 mμ. line. However, when there is much vitamin A relative to the vitamin D, the blue colour does seriously interfere. In my experience the blue colour begins to cause difficulty when the quantity of vitamin A is more than about five times that of the vitamin D.

If calciferol is the only sterol present in the mixture the problem is easy and the determination can be made with a good degree of accuracy down to small quantities. Unfortunately the orange or pink colour is given in greater or less degree by various sterols and some polyenes. The literature is somewhat confusing and, as one does not know just what sterols may be present in an unknown material, it is difficult to apply any general correction. Those sterols other than calciferol that do give a colour with acetyl chloride - antimony chloride all give a less intense one, so there is a practical possibility of establishing blanks applicable to

particular types of material. Measurement of the absorption of some of the sterols gave the following extinctions—

	$E^{1\%}_{1cm.}$ at 500 mμ.
Calciferol	1800
Sitosterol	nil
Phytosterol (B.D.H.)	nil
Cholesterol	80
Ergosterol	300

It will be seen that each is small compared with that of calciferol, and for such a quantity as 10 μg., which is convenient for vitamin D_2 or D_3, in a total volume of 2 ml., the extinction will be—

	$E^{1\%}_{1cm.}$ at 500 mμ.
Calciferol	0·90
Cholesterol	0·04
Ergosterol	0·15
Phytosterol	0·00

As the composition of a sample in terms of its sterols is not always known, it becomes necessary to establish data on the basis of the common fats. A few such results may be given; they refer to the unsaponifiable matter calculated on the original sample, *i.e.*, 1 per cent. of the oil in the mixture after addition of the antimony chloride reagent.

	$E^{1\%}_{1cm.}$ at 500 mμ.
Arachis oil	0·05
Butter fat	0·05
Castor oil	0·06
Cocoa butter	0·025
Cod-liver oil	0·057
Olive oil	0·033
Tea-seed oil	0·025

It will be noticed that margarine is not included; this is because its composition is variable and generally unknown and is likely to include large proportions of fish oils which themselves vary greatly in their content of unsaponifiable matter and its constituents.

It has been shown by Henry and Kon[3] that cows' butter maintains a constant level of about 0·07 to 0·1 I.U. per gram in the winter and 0·55 to 0·97 I.U. during the summer. These quantities would account for an extinction of only about 0·002 on 1 gram, which is much too small for satisfactory measurement and would be overshadowed by irrelevant absorption. It is not practicable to measure less than about 50 or 100 I.U. per gram.

It appears at present that the spectrophotometric method can only be applied when the composition is known or may be assumed. The method has distinct usefulness as a control on manufacture, as once the necessary blanks have been set up it is only necessary to observe the extinction under standardised conditions and ensure that the total E value does not fall below a pre-determined figure. The Public Analyst is not always in the happy position of knowing what is present or of having a good blank. He can, however, establish a maximum. Clearly the vitamin D cannot be more than is indicated by the value $E^{1\%}_{1cm.} = 1800$ and is almost certainly less, unless the mixture under examination contains calciferol but no animal oil. If the oils or fats present are known or can be determined, then the extinction due to them can be estimated within a margin of error and allowance made.

This method has been applied to the control of certain vitamin products now enjoying a considerable sale and it has been found to work. The amounts found have generally agreed reasonably well with the amounts of vitamin added by the manufacturer. For example, the unsaponifiable matter extracted from 3 g. of a sample of a well-known product containing about 33 per cent. of cocoa butter. The extraction of the unsaponifiable matter was carried out as for a cod-liver oil with all due precautions against atmospheric oxidation. The unsaponifiable matter was dissolved in 10 ml. of *cyclo*hexane. To 0·2 ml. of the solution was added 1·8 ml. of the acetyl chloride - antimony chloride reagent at a temperature of 20° C. The colour was at first bright blue, but the blue soon faded and a pale orange tint remained; it is necessary to use a quantity of the sample such that the blue colour disappears within 4 or 5 minutes. The mixture was transferred to the 1-cm. cell of a Spekker spectrophotometer and the extinction measured after 6 minutes, using the stages 0·02, 0·04, 0·06 . . . 0·30. From the observed value is deducted the appropriate blank, which is usually about 0·03.

If 3·0 grams were taken, the final concentration is 3 per cent., so the amount of vitamin D is: (observed E − blank) × 600 I.U. per gram.

It sometimes happens that the amount of vitamin A is inconveniently large in relation to the vitamin D. This is shown by the blue colour being so intense that it does not fade sufficiently within 5 minutes for a clear reading to be obtained, while if a smaller quantity be taken the colour due to vitamin D is too faint for satisfactory measurement. In these circumstances I have applied the chromatographic method devised for whale liver oils by Gridgeman, Gibson and Savage.[4] All the vitamin A can be removed and the cholesterol-calciferol fraction afterwards washed out with petroleum spirit and ether. This enables a satisfactory reading at 500 mμ. to be obtained if it is known that there is no substantial amount of cholesterol present.

It remains to be mentioned that certain other reactions have been proposed, *e.g.*, with glycerol dichlorhydrin containing acetyl chloride (by Sobel, Mayer and Kramer[5]), which are said to be more specific for vitamin D and to be unaffected by cholesterol. However, the extinction curves have a less well-designated peak and the method has not been extensively studied.

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Discussion

Dr. J. Green said that the reagent must be meticulously prepared and be quite pure, or very erratic results would be obtained. With oils containing usual amounts of vitamin A or products containing irradiation intermediate sterols or 7-dehydrocholesterol, direct estimation fails and separation techniques must be used.

Dr. Cox, in reply, agreed with Dr. Green that the method was not applicable to oils containing relatively large proportions of vitamin A, or to whale oils and products containing unknown sterols. The method he was putting forward should only be applied to known products or mixtures when the necessary blanks and controls could be established. He agreed as to the need of preparing pure alcohol-free reagents.

The Testing of Atmospheric Conditions in Theatres and Cinemas

By J. F. CLARK

(*Read at the meeting of the Society on Wednesday, May 3rd, 1950*)

Synopsis—The terms on which theatre and cinema licences are granted usually include some requirements regarding the ventilation, and an investigation has been carried out to test the adequacy of the conditions laid down in the licences.

Theatres and cinemas were inspected at peak periods. Temperatures, humidities and carbon dioxide concentration were determined at several points in each. It was found that temperature and humidity varied more with weather conditions than with the internal atmosphere, but the carbon dioxide content of the air was a reliable indication of the ventilation. In particular it was noticed that "stuffiness" became apparent to the observer at a carbon dioxide level of 15 to 20 parts per 10,000.

A simple method of sampling and testing for carbon dioxide concentration, accurate to 0·2 part per 10,000, was developed. Rapidity of sampling was essential because not infrequently adjustments of ventilation were made while sampling was in progress.

With most systems of ventilation it was considered that the usual requirements of a maximum of 10 parts per 10,000 was too stringent, since to maintain this level near draught screens and in high balconies an air supply that produced pronounced draughts in the more open parts of the auditorium was required.

Ventilation giving a maximum of 15 parts of carbon dioxide in the least ventilated areas resulted in an average concentration of under 12 parts throughout the auditorium (corresponding to a rate of 700 to 800 cu. ft. of fresh air per head per hour) and this could be maintained by any efficient ventilating system without producing noticeable draughts.

Cinemas and theatres are subject to licensing and control by local Justices of the Peace and in laying down the conditions under which a licence is granted an attempt is usually made to ensure that an adequate standard of ventilation is maintained. During a review of these conditions of licence it was decided to investigate the practicability of the Justices' requirements.

The standard of comfort and hygiene of an auditorium atmosphere depends on many factors, including temperature, humidity, outside weather conditions, arrangement of seating, supply of fresh air and its velocity, and suspended impurities; for control purposes it is desirable to have some index of an easily determinable nature to show the state of the air. It is not proposed to enter into a discussion of the whole question of ventilation here. That was very ably done some time ago by R. C. Frederick at an Institute of Chemistry lecture, and the monograph[1] is available to anyone wishing to follow up the subject.

We might briefly define an undesirable atmosphere in the circumstances we are now considering as one that has been too much used. It will be hot, humid, hazy (if smoking is permitted) and will contain too high a proportion of the products of respiration and, not infrequently, perspiration also. Such an atmospheric state is the result of an insufficient supply of fresh air, but an excessive supply, although more hygienic, may be almost as unpleasant if it results in a cold draughty hall. The object of adequate ventilation is to strike the mean between draughts and "stuffiness."

The optimum temperature is a matter of personal preference and is relative to outside weather conditions and other factors. It is a matter of comfort more than health, and so may be regarded as something to be arranged by the manager to suit his patrons in competition with similar establishments. It should, however, be borne in mind that it is cheaper to use the bodily heat of an audience to warm the atmosphere than to supply warm air. This will be referred to later.

Humidity is influenced both by the audience and by outside conditions, and also by

the fact that in many modern buildings the air drawn in is washed by passing through a water spray. The katathermometer, by measuring rates of heat loss, gives a very useful indication of the combined effect of temperature, humidity and air flow, but unfortunately it is not a suitable instrument for operating in crowded cinemas in the dark, so that it was decided not to use it in the investigation. An interesting possibility in this direction would be the use of one of the "drinking birds" which appeared as toys a year or two ago. If such a device could be standardised or calibrated, it would provide a very simple wet kata, and it would only be necessary to take the rate of dipping.

A large number of tests were made with a whirling hygrometer, from which it was found that the relative humidity was lower inside a building than outside, owing to the higher temperatures, but there was little consistency in the results. Calculated as the percentage of water vapour in the air, the humidity ran parallel with the atmospheric conditions on any particular occasion, but no correlation was found possible in the results from different theatres on different days. The method was discarded with regret, as it seemed a very suitable one by which managements could have checked the efficiency of ventilation for themselves.

Respired air, of course, contains more carbon dioxide and water vapour than fresh air, and also contains in suspension moisture droplets which can carry infection. The hygienic reason for ventilation is the need for the removal of these droplets and the associated bacteria, and also the avoidance of the effect of high temperature and humidity on respiratory surfaces, which are rendered less resistant to infection.

Since the attempt to assess ventilation conditions by humidity measurements proved unsuccessful, owing to the many contributory factors, the accepted method of using the carbon dioxide concentration as an index of the extent to which the air had been used was investigated. It cannot be regarded as more than an indicator, as in the worst ventilation conditions the amounts found are of no toxic significance. One of the first symptoms of an excessive carbon dioxide concentration is a diminution of the critical faculties and a tendency to hilarity, which might not be disadvantageous at times in theatres and cinemas.

The normal carbon dioxide output of a man at complete rest is just over 0·5 cu. ft. per hour, which can increase to 2 or even 3 cu. ft. with heavy exertion. For women and children the amounts are less. The average output of an audience under normal conditions was taken as 0·6 cu. ft. per person per hour, and on this basis, knowing the carbon dioxide contents of the fresh air supply and the air in the auditorium, it is possible to calculate the rate of supply of fresh air in terms of cubic feet per head per hour.

Working on these lines, the testing of ventilation resolves itself into problems of sampling and estimation of carbon dioxide. A reasonable size of sample is desirable, to cover a small area rather than a single point, as the latter might consist of almost undiluted exhaled air. It should be taken at about the height of the faces of the audience, and with as little noise and fuss as possible, since the most useful time of sampling is when the house is full and settling down, and distracting circumstances are then unpopular. It must be taken fairly rapidly, in view of the fact that some economically-minded managers stop the fans for a while after the house has refilled, to allow the theatre to warm up from the body heat of the audience. In such instances, the immediate reaction to the knowledge that tests are being made is generally a speeding up of the ventilation fans.

Experimental

First tests were carried out with absorption trains using various absorbents, but the amount of air to be passed to give a weighable amount of carbon dioxide necessitated too rapid a flow for efficient absorption. A small calibrated electric pump was useless, in view of the varying resistance to gas flow in the tubes. More success followed the use of a standard hand pump drawing 125 ml. per stroke, but this had to be used very slowly. The apparatus was cumbersome and attracted undue attention. With too slow a stroke, the full 125 ml. was not drawn, and too rapid a stroke meant incomplete absorption. In the latter event, with solid absorbents, a loss in weight was recorded in some instances, possibly because of the removal of fine particles of absorbent from the tubes by the pumping.

In view of these difficulties, the attempt to combine sampling and estimation in one operation was discontinued, and sampling alone was done on the site. This was accomplished most successfully by blowing up football bladders, which, when fitted with well-greased glass stopcocks, proved most useful gas containers. They can be filled quickly and, if in good condition, will take 4 to 5 litres of air and hold it without determinable change for several

days. When exhausted they contain only 3 or 4 ml. of residual air. Some apprehension was felt about the possible condensation of moisture inside the bladders and at first they were pumped up through a tube containing anhydrone, but this introduced complications, as allowances had to be made for loss in volume of the gas on drying. It was found that condensation did not take place in the bladders if extreme changes in temperature were avoided.

APPARATUS—

A simple apparatus, shown in Fig. 1, was devised for the estimation of the carbon dioxide, and consisted of an aspirator and a bubbler. The aspirator was an ordinary 5-litre bottle with a pressure tube so that the internal gas pressure could be observed and adjusted to atmospheric. The bubbler consisted of a large test tube with a centre tube, blown into two bulbs, fitting fairly closely inside it. The absorbent was barium hydroxide, roughly 0·1 *N*, containing about 5 per cent. of barium chloride. The effect of this type of bubbler is that the gas is held below and between the bulbs in contact with the liquid and released in a stream of bubbles through two layers of absorbent. With the carbon dioxide concentrations experienced and rates of flow not exceeding 2 litres per hour, it was found that one bubbler was quite sufficient and no carbon dioxide was absorbed in a second tube connected in series.

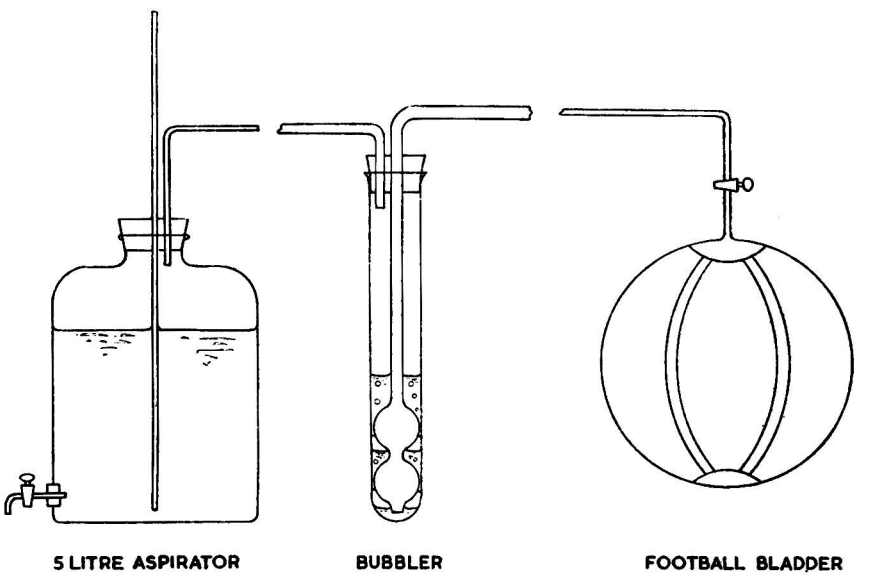

Fig. 1

METHOD—

Ten millilitres of baryta were used in the tube and in a "blank" tube of the same type, kept closed during the passage of the air; the same pipette was used to measure into both tubes. After passing the air, the contents of the tube and the blank were washed into flasks with carbon dioxide-free water and titrated with oxalic acid adjusted to such a strength (approximately 0·084 *N*) that 1 ml. was equivalent to 1 ml. of carbon dioxide gas under room conditions of temperature and pressure. Phenol thymol phthalein was found to be the most suitable indicator, with a good colour change and sensitive to 0·02 ml. The titration gave a direct reading of the volume of carbon dioxide in the volume of air passed.

The procedure was to connect the bubbler to the aspirator and the bladder to the bubbler. The bladder stopcock was partially and cautiously opened, and when the aspirator was in equilibrium with the bladder (at a pressure of about 30 cm. of water) the water was drawn from the aspirator into a measuring cylinder at a slow trickle. The control of pressure from the bladder enabled a very steady gas flow to be maintained. When about 2 litres had been drawn off (or less, if barium carbonate was seen to be forming quickly) the bladder stopcock was closed and the water stream continued until the pressure in the aspirator was atmospheric. The volume of water collected was taken as the volume of gas passed at atmospheric pressure.

It was actually very slightly higher, owing to the amount of water vapour picked up by the gas on passing through the bubbler, but the difference is well within the experimental error.

At all the theatres and cinemas, samples of street air were taken and, although on the outskirts of the town, or in the town itself on breezy days, the carbon dioxide concentration was steady at 3·2 to 3·5 parts per 10,000, on still days in the town it sometimes rose as high as 6 per 10,000. These figures were taken into account in assessing ventilation efficiency.

RESULTS AND CONCLUSIONS—

It was concluded from the investigation that the usual limit of 10 parts per 10,000 in any part of the auditorium (necessitating an air supply of 1000 cu. ft. per head per hour in the most sheltered areas) was too stringent to be effectively operated, as it involved violent draughts and an average carbon dioxide concentration of 7 to 8 parts per 10,000 throughout the theatre. With a concentration of 5 to 6 parts per 10,000 in the outside air, this level is impracticable.

It was decided that a practical limit which could be reasonably enforced was 15 parts per 10,000 in any part of the auditorium. In a modern cinema, when this level is reached in the least ventilated areas, the average concentration is found to be below 12 parts per 10,000, corresponding with an air supply of 800 cu. ft. per head per hour. It was found that 15 parts per 10,000 was the approximate point where "stuffiness" became noticeable on entering from outside, although acclimatisation was very rapid. After a little experience it was quite possible to tell on going into a cinema whether the concentration was above or below this point.

The main ventilation systems found in public halls are generally either "plenum" in the more modern buildings, or exhaust, in the older ones. They can be roughly distinguished as "push" and "pull" systems. In the plenum cinema arrangement, air is drawn through a fine wire mesh, washed by a water spray, and injected through grilles alongside the screen. It is exhausted through grilles situated under the balcony, in the roof at the back of the balcony and often high up on the back and side walls. The air distribution is good and few dead areas are found, these being generally in front of draught screens and at the back of the balcony. With such systems there is little difficulty in keeping the carbon dioxide content below the suggested limit, even with a packed house. Inefficient ventilation in such buildings means that the fans have been slowed down or stopped, which may have been done to allow the house to warm up.

With the exhaust system, air is admitted or finds its way through doors, windows or grilles, and is withdrawn by exhaust fans from the walls or roof. Dead areas are plentiful, particularly in the centre of blocks of seats and under balconies, and there is considerable difficulty in maintaining a good atmosphere with a full house.

Some results from typical cinemas with these systems may be of interest. A cinema with an exhaust system was inspected at 9 p.m. one evening when the house was full, and concentrations of carbon dioxide ranging from 21 to 25 parts per 10,000 were found (outside air 3·5). Next morning, with a wind blowing and all the doors and windows open, the air on the ground floor was at the same concentration as the street air, but at the back of the balcony there was still 11 parts per 10,000. The same evening, again with a full house and the fans at full speed, the results ranged from 13 to 18 parts per 10,000—obviously an unsatisfactory system.

A plenum system cinema, with a full house, gave figures from 12·1 to 17·6 (outside air 3·3). Next morning, the doors having been kept closed, the atmosphere was the same inside as outside the cinema. Re-inspected by arrangement with the management, the carbon dioxide ranged from 9·7 to 10·7, showing that the system was quite efficient. Samples taken from the inlet and exhaust ducts showed that the washing did not affect the carbon dioxide content of the incoming air, although it removed a surprising amount of dirt. In the main extraction duct the concentration was 11·3 parts per 10,000 and in one from under the balcony, 8·9. The hand pump and bladders proved very convenient for taking air samples from ducts and otherwise inaccessible places.

A final point concerns a question which is often raised—the effect of smoking on the carbon dioxide content of the atmosphere. The amount of carbon dioxide produced from one cigarette is of the order of 0·02 cu. ft., and it is doubtful if the consumption of cigarettes exceeds on the average one per person per hour. Thus the additional carbon dioxide produced

by smoking is of the order of 3 per cent., which is far less than the individual variation in expired air. Much more significant than the increase in carbon dioxide is the haze produced, which is composed of minute droplets of tarry matter. In the darkness of a cinema this is kept down to a satisfactory level by the suggested ventilation rates, but it is worth remarking that countries which allow smoking in cinemas are, in my experience, in the minority. In well-lit theatres and concert halls where smoking is permitted, ventilation can almost be left to look after itself, since any system which will keep the atmosphere satisfactorily clear of tobacco smoke will ensure that the concentration of carbon dioxide is well below the suggested maximum limit.

Reference

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City Analyst's Department,
Mount Pleasant, Liverpool, 3.

Discussion

Mr. C. H. Manley said that, with regard to the question of smoking in cinemas, he understood (and he noted that Mr. Clark agreed) that in Australia smoking was forbidden in the auditorium, there being a half-time interval during which patrons could smoke and partake of refreshments.

Dr. H. E. Cox noticed that the author expressed the opinion that the proper limit for carbon dioxide was about 15 parts per 10,000, and he wondered whether it could really be valid to arrive at such a figure on a chemical basis alone. What would the physiologist say? He thought the limits both of carbon dioxide and moisture needed to be related to their physiological effects. Much data on this had been accumulated in relation to submarines. The present problem should be studied in the light of experience in mines and submarines.

Mr. J. G. Sherratt referred to the type of carbon dioxide absorption tube used by Mr. Clark and asked by what method the author had proved that it was fully effective. In the questioner's experience there was risk of incomplete absorption because of the relatively large volume of air enclosed within each bubble, and he had discarded this type of tube in favour of one utilising glass beads moistened with barium hydroxide solution.

Mr. F. L. Okell, in reply to Mr. Sherratt, said that the efficiency of a bubbler of the type used by Mr. Clark did not depend on the size of the gas bubbles, but on the narrow space, about half a millimetre, between the wet walls of the bulb and the outer vessel through which the gas had to pass in a thin layer. Between each "bubble," the surfaces of the gas passage were re-moistened by the absorbent and the result was very efficient absorption. The original designer was G. Nevill Huntly, who was for many years a well-known member of the Society.

Mr. Clark, in reply to Dr. Cox's point, said that the carbon dioxide had no physiological significance in the concentrations ordinarily found in theatres and cinemas. It merely served as an indicator of the ventilation by showing the extent of dilution of exhaled air. The suggested limit of 15 parts per 10,000 had been decided on as a reasonable and attainable compromise between over-ventilation causing draughts and under-ventilation causing stuffiness. Mr. Frederick's paper, to which he had referred, went much more thoroughly into the physiological effects of extremes of ventilation, and the data there had mostly been accumulated in connection with Admiralty work, with particular reference to atmospheres in confined spaces.

His experience confirmed Mr. Okell's statements regarding the efficiency of the bubbler. If the bulbs of the bubbler fitted closely to the sides of the tube, a second bubbler in series did not absorb a determinable amount of carbon dioxide, and a known amount of carbon dioxide was completely recovered within the limitations of accuracy of the method. The bubbler had been successfully used for other carbon dioxide estimations such as the testing of self-raising flour and baking powder. It was, however, inadvisable to exceed about two-fifths of the absorptive power of the baryta.

Hormone Assay

The following four papers were read at a joint meeting of the Biological Methods Group and the Society for Endocrinology on Thursday, October 20th, 1949.

Introduction

By J. H. GADDUM

CANDIDATES in pharmacological examinations are sometimes asked why certain substances are assayed by biological methods. The correct answer is, of course, that no one would do bio-assays if he could help it, but that some drugs, such as insulin, cannot be controlled in any other way, and some drugs, such as acetylcholine, are commonly present in concentrations that are too low to be detected in any other way.

The motives that drive us to bio-assays can, however, also be classified pragmatically. Some of us wish to estimate the potency of commercial preparations, so that they may be safe to use and so that we may assess their commercial value. For these purposes, methods of bio-assay have been developed that can give almost any required degree of accuracy. The members of the Biological Methods Group of the Society are expert in such matters. The members of the Society for Endocrinology, on the other hand, are interested in bio-assays mainly as a means of discovering new facts. Their problems are complicated and their arguments often involve assumptions and approximations that are open to criticism. The object of both should be that the endocrinologists should learn accuracy and that the analysts should learn something of the difficulties of the endocrinologists.

Methods of hormone assay can be classified according to the kind of effect observed, though some hormones can be assayed by means of several different kinds of effect. Table I summarises most of the established methods of assay for hormones and gives some indication

TABLE I

	Smoked drum	Animal weight	Organ weight	Fluid volume	Bio-chemical	Mor-phology	Death, etc.
Adrenaline, etc.	1				1		
Thyroid		1			1	2	1
Androgens			1				
Oestrogens			1			2	
Progesterone						1	
Corticoids					1	2	1
Insulin					1		2
Parathyroid					1		2
Secretin				1			
Oxytocin	1						
Vasopressin	1						
Antidiuretic				1			
Growth		1				2	
Prolactin			1			2	
Thyrotrophin			1		1	2	
Gonadotrophins			1			2	
Corticotrophin					1	2	
	1 quantitative.			2 quantal.			

of the effects. Most of the tests are based on a quantitative measure of the effect. A few of these are quantal in the sense that the response of each animal is not measured, but merely recorded as positive or negative. These latter are denoted in the table by the figure 2; other things being equal, they require for any given degree of accuracy about twice as many animals as the first type of test.

Under the heading of morphology are included observations of the presence of legs in tadpoles, of the appearance of vaginal smears and of histological preparations of sections of tissues. Such results are difficult to assess quantitatively and the test is generally quantal.

The heading, "death, etc.," includes the symptoms produced by insulin in mice. It may be asked how death can be quantitative. The answer is that some tests depend on the duration of survival.

The last three hormones in the list act primarily on other glands. For example, when gonadotrophins are administered to males they act on the testes, which release androgens, and these androgens then act on the prostate. It might be expected that assays based on the weight of the testes would be more accurate than assays based on the weight of the prostate, but this is not necessarily so.

We probably agree about the principles upon which assays should ideally be founded—

(1) Each assay should involve a comparison between an unknown preparation and a standard preparation.

(2) Each of these preparations should contain the same single active principle and no other substances that modify its action in the test.

(3) Each assay should be complete in itself; it should involve no arbitrary assumptions about such things as the slope, or even the shape, of the dose - effect curve.

(4) The limits of the error should be calculated from the internal evidence of each assay.

If the first two conditions are fulfilled, the test must give an estimate of the concentration of a single substance, and the results of parallel quantitative tests of the same solution by a series of different methods will all agree, within the errors of the tests. If the substance used as a standard is not the same as the substance present in the unknown solution, it is generally found that the results of parallel quantitative tests do not agree. This circumstance forms the basis of a valuable method for identifying substances and distinguishing them from other closely related substances. By means of such tests it has been shown in recent years that adrenaline is generally accompanied, or replaced, in the body by the closely related substance noradrenaline.

It follows from these facts that the biological response on which the result of an assay depends need have no relation to the effect that the preparation is expected to produce.

The requirements of an ideal assay are seldom, if ever, completely fulfilled. Even in careful assays of insulin the assumption is generally made that the dose - effect curve can be transformed into a straight line by mathematical devices that have been successful in the past. In practice, it is necessary to compromise in order to make any progress at all. We may discuss how far this compromise should be allowed to go.

A standard preparation is not always obtainable. There is no international standard preparation for many of the hormones, and in many instances it is not yet clear how to make a standard preparation. A later paper will tell, for example, something about human pituitary gonadotrophins, which are only available at present as variable mixtures of unknown substances that increase one another's effects to unknown extents and cannot be separated from one another.

Oestrogens should present a simpler problem since their chemical formulae are known, but even so it is impossible at present to make satisfactory assays of mixtures of oestrogens. The reasons for this have been discussed fully by Emmens,[1] but let us consider some of the facts.

Biological fluids may contain variable proportions of three oestrogens, oestradiol, oestrone and oestriol. The first of these substances is the most active and the last the least active, but the ratios of the activities depend on the details of the experimental technique and may vary enormously. For example, Emmens quotes figures varying from 1 to 250 for the ratio of the activity of oestrone to that of oestriol. In his own experiments, the ratio was 2·4 when the drugs were given in four doses, and 70 when they were given in two doses. These figures alone are enough to show that it is impossible to attach any real meaning to the results of assays of unknown mixtures of oestrogens. Estimates which purport to give "oestrogenic activity" of such mixtures are liable to vary over a 30-fold range or more, according to the way the test is done. Consider an imaginary example. A control sample of urine contains 1 mg. of oestrone and 10 mg. of oestriol. The patient is then given a dose of medicine, and another sample of urine is collected containing 2 mg. of oestrone and 4 mg. of oestriol. Two observers both estimate the oestrogenic activity of these samples of urine in terms of oestrone. Observer A gives the oestrogens in two doses and observer B gives them in four doses. In the first test, according to Emmens, the ratio of activity of oestrone to activity of oestriol is 70, and it may be calculated that the conclusion will be that the medicine causes

a *rise* in the oestrogenic activity of the urine from 1·14 mg. of oestrone equivalent to 2·06. In the second test, the ratio is 2·4, and the conclusion will be that the medicine causes a *fall* in the oestrogenic activity from 5·17 to 3·67. The two methods give opposite results and they are both wrong; their results have no meaning.

The variation of ratios can, of course, be reduced by standardising the technique, but if this is done the interpretation of the results becomes very artificial. In the example just discussed, any conclusions about the effect of the medicine would depend on which system of doses had been arbitrarily chosen as the standard one. The position is further complicated, as Emmens pointed out, by the presence in urine of variable quantities of substances, not oestrogenic themselves, but having the property of increasing the effect of substances that are.

The discovery of the synthetic oestrogens has made it unnecessary to use biologically standardised mixtures of oestrogens in therapeutics. If it had not been for this discovery, as much time and labour might have been wasted in the study of methods of standardisation of mixtures of oestrogens as has been wasted on the same problem in connection with extracts of digitalis. These are known to contain a variable mixture of active substances, and enormous amounts of scientific energy have been devoted to the search for a reliable method of predicting their effect on man. Such experiments serve a practical purpose, but rest on an insecure logical basis and scarcely deserve the name of assays at all. It is hoped that they will soon become unnecessary because doctors will use pure active principles.

The only possible way of getting results with any intelligible meaning in bio-assays of mixtures of oestrogens seems to depend on removing the interfering substances and separating the oestrogens from one another before the biological tests are made. Chemical methods give results that are easier to interpret, since the three main oestrogens have equal effects in the Kober test generally used, so that it is possible to estimate the total amount of oestrogen present. This test is, however, not sensitive enough to estimate the oestrogens in non-pregnant blood. If a biological test could be discovered in which all the oestrogens were equally effective, progress could be made, but sufficiently sensitive chemical methods would be even more valuable.

Accurate tests for single oestrogens have, of course, been available for years, but they have played only a small part so far in advancing knowledge. The researches that have led to our present detailed information about the chemistry of these substances were controlled by biological tests in which all the laws of bio-assay were neglected. This shows that it sometimes pays to take risks, but does not justify all the risks that have been taken by endocrinologists. In some instances it may still be necessary to speak of mouse units and to assume that all the mice in the world are equal, but every effort should be made to get away from such dangerous assumptions as soon as possible. There can be no justification for speaking, as some still do, of mouse units of the chorionic gonadotrophin present in the urine of pregnant women. An international standard preparation of this substance is available and should be used. Results should be given in international units. Unfortunately, this standard cannot be safely used for assays of other gonadotrophins, such as those present in the urine after the menopause.

Apart from the provision of reliable preparations of hormones, the contribution that bio-assays can make to clinical endocrinology depends on the estimation of hormones in blood and urine. The concentration in the blood presumably gives an indication of the concentration with which the tissues are in equilibrium. The concentration in the urine may provide an indirect estimate of the daily output of hormone, provided that all the urine excreted during 24 hours is collected. If, for example, it is found that 5 per cent. of a hormone appears in the urine when it is injected, it may be justifiable to make the provisional assumption that the daily output by the gland is equal to the amount excreted in the urine multiplied by 20. In such instances it is important to remember that changes observed in the urinary output may represent either a change in the amount of hormone released into the blood stream or a change in the proportion of the hormone reaching the urine. So long as this fact is borne in mind, estimations on the urine may be expected to give valuable information not given by estimations on blood.

The time will probably come when provision for assays of this type will be an essential part of the equipment of all large hospitals, but that time is not yet. Very few methods of assay available at present are sensitive enough for this purpose; the discovery of more sensitive methods is a most important objective. Chorionic gonadotrophin is present in such high concentrations during pregnancy that its concentration in both blood and urine

can be accurately estimated by bio-assay, but this is not true of any other hormone. Until more sensitive tests are discovered the amount of hormone available is too little for a perfectly designed assay.

REFERENCE

1. Emmens, C. W., "Variables affecting the Estimation of Androgenic and Oestrogenic Activity," Spec. Rep. Ser., No. 234, Medical Research Council, London, 1939.

PHARMACOLOGICAL LABORATORY
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EDINBURGH, 8

General Approach to Biological Assays

BY H. O. SCHILD

SYNOPSIS—The term "bio-assay" is used for two different procedures. In the first, an unknown sample is assayed in terms of a standard of the same composition, the aim being to determine the amount of some chemical substance present; this may be termed "analytical assay." In the second, the test sample is assayed in terms of a substance or mixture of different composition, the aim being to determine the biological activity of the test sample. The paper briefly reviews some of the principles underlying the analytical type of assay.

BIOLOGICAL assays may be used for two purposes, which must not be confused. Either they may be used analytically, as substitutes for a quantitative chemical analysis, or they may be used to determine the biological activity of a substance or a mixture of substances.

In the first case the aim of the assay is to detect the concentration of a certain chemical substance in a sample. The only essential condition is that the standard and unknown should have the same composition. If they are chemically identical, then it does not matter what species or what reaction is used in the test; the final result is always the same and the best method is that by which an accurate result is produced with the greatest economy of time and labour.

An entirely different situation arises where the two samples differ in chemical composition. The assay then ceases to be an analytical method and becomes a comparison of biological activity in which species, end-point and experimental conditions become all-important.

When two substances that are chemically different, say cocaine and procaine, are compared, it is impossible to assign to them an activity ratio in general terms, since their relative activity depends on experimental conditions. Further, even under constant experimental conditions, the results of successive experiments are not necessarily identical, as the relative sensitivity of a preparation to different substances is not constant.

Not only major chemical differences, but even minor differences in composition and purity may completely distort an assay and change it from an analytical method into a comparison of activity valid only for the particular species and method of administration used. For example, Gold, Cattel, Kwit, Kramer, Modell and Zahm[1] found that by oral administration in man, 1·25 mg. of digitoxin produced the same effect as 1·25 g. of digitalis, when tested by a fall in heart rate in patients with auricular fibrillation. By the intravenous cat method, however, the former dose corresponds to 3 to 4 lethal "cat units" whilst the latter corresponds to 12·5 cat units. Although digitoxin is the main active constituent of digitalis, yet in this instance differences in purity, in the method of administration and in the species used led to entirely different results by the two methods.

COMPARATIVE ASSAYS

NECESSITY FOR A STABLE STANDARD—

It is generally believed that biological assays, at least those of the analytical kind, should be comparative and should be made by reference to a stable standard. This belief is based on two assumptions: first, that it is not possible to keep a biological reaction under

"statistical control" in different laboratories and that statistically significant variations in the activity of so-called animal units occur between different laboratories and also within the same laboratory at different times; secondly, that international standard preparations are stable and do not deteriorate.

A good example of the variability of animal units is provided by the collaborative digitalis assay done for the U.S.P. under the guidance of Bliss.[2] In this assay the intravenous lethal cat dose of digitalis was determined, using the same standard preparation in various laboratories and at various periods. The assay technique was completely standardised and in each experiment the standard was compared with an unknown consisting of a dilution of the standard preparation.

When the results were analysed it was found that the lethal dose of standard—the cat unit—would stay constant, within the limits of error of the assay, only for periods of about 8 or 10 days in any one laboratory. During that period there was therefore no need, in assaying the unknown, to refer back more than once to the standard preparation. When, however, the assay was repeated in the same laboratories a few months later, the cat unit had altered significantly in about half of the laboratories. Table I, which gives some data

TABLE I

Variation	Degrees of freedom	Mean square	Variance ratio, F
Between collaborators	9	0·03162	4·99
Between preparations	1	0·11491	18·15
Collaborators × preparations	9	0·00463	0·73
Error	160	0·00633	1·00

taken from Bliss's paper, shows that significant variation in the lethal dose occurred between different laboratories (collaborators), but it is of interest that the mean square for "interaction" of laboratories on preparations was no greater than that for error. As in this instance interaction measures the relative potency of standard and unknown as determined in different laboratories, the result signifies that although the absolute activity as determined in different laboratories varied, the relative activity of standard and unknown remained the same within statistical limits in the hands of different investigators. In other words, whilst the absolute values could not be brought under statistical control the relative activities had been so controlled. This is a good demonstration of the superiority of comparative assays over assays relying on animal units.

DETERIORATION OF STANDARD—

In using comparisons with a stable standard, rather than animal units, for biological standardisations, one assumes of course that the variation in activity of the stable standard is negligible compared with the variation of the animal unit. Unfortunately, there is no way of determining whether this is true or whether a so-called stable standard is deteriorating.

This problem arose, for example, in 1942, when the standard for posterior pituitary at Hampstead was replaced by a fresh standard. The new standard was compared in a number of laboratories against the old standard by the three methods, oxytocic, vaso-pressor and antidiuretic, and was generally found to be about 15 per cent. more active than the old one. The Biological Standards Commission, however, decided to adopt as a unit the same weight of the new standard as had been used for the old standard, taking no account of the higher activity of the new one. Both standards had been prepared by the methods of Voegtlin, who had himself assumed that an extract prepared from fresh glands strictly according to his instructions would always have the same activity. This may well have been so, and, in that event, one has to assume that the old standard had slightly deteriorated. I do not wish to suggest, however, that everyone should now prepare his own standards by Voegtlin's methods for fear that the international standard may have deteriorated. It may be expected that workers less skilled than Professor Voegtlin would produce extracts varying in activity by much more than 15 per cent.

STANDARDISATION ON MAN

In many biological standardisations one is dealing with impure extracts whose identity with the standard cannot be guaranteed. Since relative activity may then depend on the species and end-point used for the assay, should these standardisations be done on man?

The answer can only be found experimentally by comparing standardisations done on animals and on man.

The most thorough attempt at standardisation on man has probably been that of Gold, Cattel, Otto, Kwit and Kramer[3] in New York who tried to standardise digitalis on man. These workers used two reactions: the slowing of the heart rate in auricular fibrillation and an inversion of the T-wave in the electrocardiogram. The first method is preferable because it measures a therapeutic action, but the second gives more accurate results.

In one series, using the slowing of the heart rate, these workers concluded that the result of comparisons for activity of the standard powder with an unknown in man agreed fairly well with those of the cat assay, although the agreement between the human assay and the frog assay was not as close. In another series, however, using the T-wave test, they found marked discrepancies between the human assay and the cat assay, amounting sometimes to over 50 per cent. Apparently these experiments have not been repeated.

Posterior pituitary hormone is another interesting example. Kamm separated the oxytocic and the vaso-pressor principles, using the guinea-pig uterus as test object. Pitressin had only a very small action on the guinea-pig uterus, and this was usually attributed to contamination with pitocin. Later on it was found that pitressin did affect the uterus in some species; for instance, in the cat it may produce a strong inhibition of the uterus (Robson and Schild[4]) and more recently it has been shown by Chassar Moir[5] that in man pitressin produces under certain conditions a greater contraction of the uterus than pitocin.

Obviously a pituitary extract standardised by the guinea-pig method gives no adequate indication of activity in the human uterus. The routine assay of pituitary extract on the parturient human uterus is probably impractical, but it would be interesting to know whether at that stage an extract of pituitary standardised by the usual methods for oxytocic activity would be more active than purified oxytocin standardised in the same way.

It would therefore seem that in dealing with mixed extracts, animal standardisations are a useful safeguard rather than a reliable method of assessing activity. It is important that animal standardisations of these extracts should occasionally be checked by human standardisation, but even this is no complete safeguard, as pointed out by Gaddum (previous paper), since all human beings do not react alike.

Statistical methods

The simplest type of graded assay is one for which no statistics are required, in which the standard and unknown are matched by a trial-and-error process until they produce equal effects. This method has one great advantage: it does not depend on the assumption of a mathematical relation between dose and effect. However, it also has several disadvantages. First, it is inefficient because the preliminary effects are not utilised in the final assessment. Secondly, it is subjective, depending on the judgment of the experimenter as to what constitutes a satisfactory match. Thirdly, its experimental error cannot be determined from the assay itself but only by separate experiments. And fourthly, since the effects are matched at only one dose level, it gives no indication of whether the concentration - action curves of standard and unknown are parallel. It thus fails to show up qualitative differences between standard and unknown.

Assays that involve statistical methods are usually based on the assumption of some mathematical relation between dose and effect. Experience has shown that frequently the relation between dose and effect is expressed by an S-shaped curve when the dose is plotted on a logarithmic scale. Thus, if the reaction is of the all-or-nothing type (quantal), an S-shaped curve results if the percentage of animals reacting is plotted against log dose, whilst if the reaction is of the graded type (quantitative) an S-shaped curve results when the size of the effect is plotted against log dose.

These S-shaped curves may be variously interpreted. Usually they are interpreted as integrated normal curves when the reaction is quantal and as Langmuir adsorption curves when it is quantitative. In order to obtain approximately straight lines only the middle part of the graded response curve may be utilised. With all-or-nothing reactions the ordinates may be transformed to normal equivalent deviations or probits. The transformed curves are usually approximately linear, especially in the range above 20 per cent. mortality. Other transformations have been used, such as the logit transformation derived from the logistic curve. Finney[6] has shown that practically identical results may be obtained in an assay using any one of four different transformations.

Gaddum has outlined three characteristics for a satisfactory assay, *viz.*, (1) a linear relation between x and y over the relevant range, (2) a standard deviation (this applies to graded responses only) independent of effect and (3) a small value of s/b. The latter is a good measure of variability, since it measures the variability of response in terms of the dose. Bliss has suggested another important characteristic of a good assay, namely, that it should furnish an estimate of its variability as an integral part of the assay.

The statistics of quantal and quantitative assays have evolved on rather different lines. This is due to a mathematical peculiarity of quantal assays, the need for weighting the response. In quantitative assays, on the other hand, it can usually be assumed that all points on the curve have the same variance and therefore the same weight. Owing to this fact, Fisher's analysis of variance could be readily applied to quantitative assays with all its implications of planning experiments in such a way that sources of variation not attributable to experimental error may be eliminated from the final comparison.

Statistical methods are becoming increasingly complex, and we must leave it largely to the statisticians to work out their mathematics. Biologists should, however, keep in mind the basic assumptions on which these mathematics are founded. For example, the limits of error of an assay involving two straight parallel lines may be calculated by one of two formulae, one giving what are usually called the approximate limits of error, and the other giving exact fiducial limits. The reason why one is called "approximate" and the other "exact" is that in the derivation of one formula a mathematical approximation is used, namely, the variance of the ratio of two variables, whilst the derivation of the other is mathematically exact. It would, however, be wrong to assume that, because the formula is mathematically exact, the actual fiducial limits as calculated in an individual assay are necessarily correct. This would only be so if the assumptions on which the calculations are based were exact. These, however, we know to be only approximations; for example, we know that the assumption of linearity in quantitative assay is an approximation. One may, of course, test the data for deviations from linearity and parallelism, but lack of statistically significant deviation from linearity does not prove a linear relation in a particular assay. Thus the fiducial limits as calculated from a given experiment must always be taken with a grain of salt. This applies particularly to research work where new situations constantly arise and where the basic assumptions have not been tested by extensive previous experience.

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The Measurement of Thyroidal Activity

By G. F. SOMERS

Synopsis—After an account of methods used or proposed for evaluating thyroid activity in natural and synthetic products, this paper includes a brief description and comparative assessment of the method based upon comparing the resistance of mice to anoxia, which is reduced by thyroid treatment. Assay methods based on the antagonistic action of anti-thyroid drugs and thyroid preparations are also discussed.

The evaluation of thyroid preparations is a problem not yet satisfactorily solved. The official method of standardisation (British Pharmacopoeia, 1948)[1] is based on Harington and Randall's estimation[2] of acid-insoluble organically combined iodine. The United States Pharmacopoeia, 1947,[3] makes use of the total iodine present in organic combination. Neither method gives values in close accord with physiological results[4]; on chemical considerations the B.P. method is to be preferred, on the assumption that thyroxine is the only source of acid-insoluble iodine.

The thyroid contains two distinct organic iodine compounds, thyroxine and di-iodotyrosine; only the former is physiologically active. The relative amounts of the two compounds differ considerably in different samples of thyroid.

The method of Harington and Randall[2] appears satisfactory for those thyroid preparations in which all the acid-insoluble iodine is in thyroxine, but it is unsatisfactory for iodinated proteins. These contain a large amount of acid-insoluble iodine, of which only a small and variable proportion has thyroid activity.[5] Chemical analysis of these preparations being therefore of little value, biological assays are necessary. A modification of Harington and Randall's method was developed by Leland and Foster[6]; in this, after the preliminary alkaline hydrolysis, the thyroxine fraction is extracted with butyl alcohol. Reineke, Turner, Kohler, Hoover and Beezley[7] have claimed excellent agreement between the results of biological assays and a chemical extraction method modified from that of Blau.[8,9]

Other chemical methods suggested involve absorptiometric measurements[10] of the red colour formed with sodium nitrite in ammoniacal solution,[11] of the red colour formed with diazobenzene sulphonic acid in sodium carbonate solution,[12] or of the purple colour formed with diazotised N^1-diethylsulphanilamide in alkaline solution.[13]

Polarographic methods have also been employed.[14,15,16] They not only determine the total iodinated diphenyl ethers present, but can also distinguish between thyroxine and 3 : 5-di-iodotyrosine.

It should be remembered that the optical form of thyroxine is all-important, the physiological activity of DL-thyroxine residing largely, if not entirely, in the laevorotatory component. Thyroxine in thyroid extracts and in iodinated proteins is solely the L-form.

Biological Assays

Although chemical methods are reasonably satisfactory for standardising gland extracts, there is occasionally a need to evaluate the thyroid activity of new materials, such as iodinated protein, thyroxine analogues and other organic compounds. In testing for thyroid activity, the accepted principles of biological assay must be applied. Simultaneous examinations of a standard preparation and the test material should be made; accurate assays are only possible when the active principle of both is the same. In comparing new compounds with old, quantitative assessments should be made with caution and results obtained with one species should not be assumed to apply to another.

Methods

Numerous biological methods have been suggested for the assay of thyroidal activity; some of them bear no obvious relationship to the physiological action of thyroxine in mammals, *e.g.*, the acetonitrile test in mice.[17] No one of them has been generally accepted, possibly because of their practical difficulties and inherent inaccuracy. Most procedures have not been developed on a reasonably quantitative basis; few workers have accompanied their reports on the comparison of two thyroid preparations with information about the precision

of the estimates attainable. Where data susceptible to statistical analysis have been given, it has often been found that in fact the precision claimed has not been achieved.

The commoner methods employed will now be briefly considered.

INCREASE OF METABOLISM—

Thyroid preparations cause an increase in metabolic rate; the associated changes occurring in oxygen consumption and carbon dioxide production can be used for the estimation of thyroidal activity. Such methods are most closely related to the physiological actions of thyroid in man, but they tend to be too laborious and troublesome for routine assays. For the measurement of oxygen consumption the animal is usually enclosed in a chamber immersed in a constant-temperature water-bath.[18,19] Expired carbon dioxide is absorbed in soda-lime and the oxygen consumed is measured by a valve or from a Benedict spirometer. By this method, Wokes[20] compared thyroid extracts on guinea-pigs, and obtained limits of error, P = 0·95, of ± 30 per cent., using two dose levels of the standard and test preparations with six guinea-pigs on each dose level. The guinea-pig is very suitable for this type of assay because of its sensitivity and rapid response. The apparatus is possibly over-elaborate, and a simpler method for measuring the oxygen consumption of mice, which should be readily adaptable to the assessment of thyroid preparations, has recently been described by Maclagan and Sheahan.[21]

For the estimation of carbon dioxide production the method of Mørch[22] is usually employed. Mice are placed in a closed container through which air is drawn after previous removal of carbon dioxide with soda-lime. The carbon dioxide evolved by the animals is absorbed in a train containing standard sodium hydroxide, the amount being subsequently determined by titration with standard hydrochloric acid. According to Mørch, the method is one of the most accurate for determining thyroidal activity, but a simpler and less time-consuming method is desirable for routine assays. Gaddum and Hetherington[23] compared the activities of a number of thyroid preparations by this method, but the results were only roughly proportional to the thyroxine contents determined by the method of Harington and Randall.[2]

INCREASED SUSCEPTIBILITY TO ANOXIA—

The thyroid gland has an influence on the susceptibility of mammals to anoxia, their resistance being decreased by previous administration of thyroxine.[24,25,26,27] Smith, Emmens and Parkes[28] have developed a method of assay for thyroid preparations based on this decreased survival time of mice enclosed in jars. We have utilised the method extensively for comparing the activity of thyroxine and a number of its derivatives.[29] For calculation of the results we used the logarithms of the survival times, because the distribution within each group of the survival times themselves was skew. The relative activities were calculated by the method of Bliss and Marks[30] and errors in the slopes provided for according to Irwin.[31] The slope, b, for thyroxine sodium by the subcutaneous route was −20·8 with a standard error (P = 0·95) of ± 3·1, b being calculated as the increase in log survival time per 10-fold increase in the dose. The value of s/b = 0·35; to attain a precision of ± 20 per cent. (P = 0·95) it would therefore be necessary to employ 240 animals on both the standard and test preparations and to use three dose levels for each. The slopes were even less steep by the oral than by the subcutaneous route (b = 14·8; s/b = 0·48). The precision of the method therefore leaves something to be desired, but the assays are relatively easy to carry out.

LOSS OF BODY-WEIGHT—

The administration of thyroid to mice, rats and guinea-pigs causes a loss in body-weight.[20,22,32,33,34] Gaddum[19] found that adult rats lost weight *pari passu* with the rise in their metabolic rate. Wokes,[20] however, found wide variations in the susceptibility of mice to thyroxine and considered that large numbers would be required for an assay based on weight changes. Hutcheon,[35] using rats, showed that the slope of the line relating the loss in weight to the log dose of thyroxine was 9·1 per unit log dose with a standard deviation of ± 4·4 g. The value of s/b is therefore 0·49. The precision of the estimates is thus not good; further, the method cannot be specific, the presence of toxic factors influencing the result. There is also a wide variation in the response of guinea-pigs to a given dose, so that large numbers must be employed under carefully controlled conditions, or the results obtained will be far from precise.[34,36]

DECREASE IN SUSCEPTIBILITY TO TOXIC SUBSTANCES—

Hunt[37] observed that previous administration of thyroid by mouth increased the resistance of mice to acetonitrile poisoning. A method for assaying thyroid preparations, based on this observation, was developed by Hunt and Seidell,[38] and this was improved by Hunt[39] and by Haffner and Konyama.[33] Unfortunately, the susceptibility of mice to acetonitrile is extremely variable and is affected by numerous known and unknown factors. Gaddum and Hetherington[23] controlled these factors in some experiments, but the variation in the response of the mouse to acetonitrile alone was so great that they rejected the procedure as a possible test method. Wokes[40] found that at ordinary room temperatures the dose - mortality curve for acetonitrile became very flattened. At higher temperatures the dose - mortality curve was steeper, but thyroid no longer afforded any protection. Laland and Støa[41] obtained more favourable results with the method and considered it serviceable for routine tests, but they have not stated its precision.

INCREASE IN THE RATE OF AMPHIBIAN METAMORPHOSIS—

Gudernatsch[42] showed that a thyroid preparation accelerated the rate of metamorphosis of amphibian larvae, and caused a rapid shrinking in size, if suspended in the water in which they were living. Several of the effects on tadpoles have been utilised for testing the activity of thyroid preparations. These include: time to first appearance of the leg buds, time to produce death, length of gut, body-weight, body volume and total length. A number of Anura have been employed, *Rana pipiens* and *Rana temporaria* being mostly used. The chief difficulty with the tadpole test has been a regular supply of tadpoles; this can be overcome by the use of *Xenopus laevis* if they are bred in the laboratory.[43] As a method of assay, the use of amphibian larvae can be criticised on the grounds of unspecificity, particularly because of the wide difference in species from mammals and in the mode of administration Di-iodotyrosine and D-thyroxine are active in amphibia[43] but relatively inactive in mammals. Quantitative methods have been described by numerous workers. Wokes,[4] using tail length in tadpoles from *Rana temporaria*, obtained results giving an s/b value of 0·38, and substantially similar values are shown by the results of Deanesly and Parkes[43] using eruption of the front legs in *Xenopus laevis*.

RESTORATION AND MAINTENANCE OF GROWTH IN YOUNG THYROIDECTOMISED RATS—

The retardation of growth in young thyroidectomised rats is prevented by the daily injection of a thyroid powder suspension or thyroxine. The possibility of using the response for measuring the biological activity of iodinated caseins and other allied substances was investigated by Rowlands.[44] The method, however, as an assay procedure, presents difficulties in practice. The curve relating dose to response is so flat that a very large number of animals would have to be used, 6100 for an error of $\pm$ 10 per cent. at $P = 0{\cdot}95$. The time needed for the test, 90 days, is also too long.

ANTAGONISM OF THYROXINE TO ANTI-THYROID DRUGS—

Certain anti-thyroid compounds, such as allyl thiourea, phenyl thiourea, sulphaguanidine, thiourea and thiouracil, prevent the formation of the active thyroid principle.[45,46,47] The subsequent decrease in the amount of circulating hormone in the blood causes an increased production of thyrotrophic hormone by the anterior lobe of the pituitary; this, through its stimulating action, causes enlargement and histological changes in the thyroid gland. The hyperplasia and histological changes induced by the feeding of anti-thyroid drugs is inhibited by simultaneous administration of thyroxine, but not by iodide,[48] and the ability of thyroidal substances to reduce the enlargement of the thyroids of chicks[49] and rats[48,50] treated with thiouracil may be used for the assay of thyroidal activity. The method gives results in accord with those obtained by the standard metabolic method.[50] According to Hutcheon,[35] the method with rats is reasonably precise, the s/b value being 0·16. The chick has advantages over the rat in its cheapness, greater sensitivity, availability and ease of handling.

The anti-thyroid drugs also cause a decrease in the iodine content of the thyroid glands, which can be prevented by the simultaneous administration of thyroxine.[51] The iodine concentration in the glands can be measured chemically or by radio-active tracer techniques using ^{131}I, and can therefore be employed as an index of thyroidal activity.

Conclusions

In spite of considerable effort, no wholly satisfactory method for measuring thyroidal activity has yet been evolved and discrepancies arise in comparing biological with chemical results. While chemical methods based on the estimation of acid-insoluble iodine (British Pharmacopoeia, 1948) may be preferred to those based on total iodine, neither appears to give a reliable indication of the physiological activity of various thyroid preparations.[4,23] Discrepancies may be large, and indeed evidence is accruing that the published estimates for the thyroidal activity of iodo-casein preparations, which has been stated to be equivalent to about 3 per cent. of L-thyroxine,[52] were in error.[53] The chief difficulty with biological assays is the extent of variation in the response of the individual animals, which necessitates the employment of relatively large numbers before an adequate degree of precision can be attained. While thyroxine is undoubtedly the active principle present in thyroid extracts[54] and in iodinated proteins,[55] the form in which it is administered plays an important part in its activity, especially by the oral route.[56,57,58,59] Differences in the availability of thyroxine in various preparations may account for the discrepancies. Similarly, the same calorigenic substances may be utilised differently by different organisms and results obtained with one species may not apply to another. We can say that neither chemical analysis nor physiological assay will give a true index of the activity of a preparation on man; this can only be settled by clinical trial. In any event the development of a new method for the synthesis of L-thyroxine[60] may reduce considerably the need for bio-assays.

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DISCUSSION

DR. H. O. J. COLLIER said that for the test of thyroidal activity, using protection against toxic agents as a criterion, it might be expected that certain toxic agents would be more suitable than others. For example, some of the substances that were known to undergo a marked chemical detoxication in the body might be suitable for this test, since the test material could be expected to stimulate the general level of activity of the cells.

Had Dr. Somers conducted any comparative experiments on different types of toxic agents for use in this test of thyroidal activity, and, if so, did they lend any substance to the foregoing suggestion?

DR. SOMERS replied that other chemical substances could be and had been tried, but he had only used acetonitrile. He thought Dr. Collier's suggestion a reasonable one that probably explained the mode of action of thyroxine in lowering the toxicity of acetonitrile.

MR. W. A. BROOM said that in his laboratory they had had experience with three of the methods described by Dr. Somers, namely, the method based on the change in metabolism in guinea-pigs, the closed vessel mouse method and the tadpole method. In their hands, the metabolism method was most unreliable and time consuming, and the mouse method, though workable, was less precise than the tadpole method. They preferred the tadpole method, which gave results that agreed very well with the experience of veterinary workers studying the effect of iodinated casein on the milk yield of cows.

DR. R. PITT-RIVERS pointed out that experimental evidence was available that D-thyroxine was active when tested on myxoedematous patients; it had one-eighth to one-tenth of the activity of L-thyroxine.[1] She did not consider it justifiable to compare a D- with a DL-amino acid and to deduce the relative activities of D- and L-forms because, in the presence of the natural isomer, the very slight activity of the unnatural isomer might be masked or lost owing to experimental error.

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The Assay of Posterior Pituitary Lobe Extracts

By G. A. STEWART

Synopsis—A review is given of the methods of assaying the oxytocic, vasopressor and antidiuretic hormones of posterior pituitary lobe extracts.

The difficulties of assaying the oxytocic hormone by the guinea-pig uterus method are described in detail, and descriptions are given of the rat uterus and chicken blood-pressure assays, which enable the hormone to be determined with a much greater degree of accuracy. Assays of the vasopressor hormone on the blood pressure of the spinal cat and anaesthetised dog are described, and mention is made of the more recently developed assays of this hormone on the blood pressure of the rat. Assays of the antidiuretic hormone on the rat and dog are described.

The relative merits of the assays referred to are compared and discussed. The variability of commercial extracts in respect to their contents of these three hormones is considered.

Three of the active principles of posterior pituitary lobe extracts are used clinically, namely, the oxytocic, vasopressor and antidiuretic hormones, and of these, as a rule, only the oxytocic and vasopressor hormones are assayed commercially. Generally an extract containing all three hormones is assayed for its oxytocic activity only.

The assay of the oxytocic hormone—

The "in vitro" method of Dale and Laidlaw[1] using the guinea-pig uterus for the assay of the oxytocic principle is beset by many practical difficulties, notably that of obtaining a suitable uterine preparation. The uterus usually employed is that from a virgin guinea-pig in dioestrus. Some uteri contract very slowly when stimulated by posterior pituitary lobe extract, some fail to give good differentiation between graded doses of the same extract, whilst others may react to the same dose of the pituitary extract with alternate large and small contractions.

As a result of these many difficulties, different workers have sought to find conditions under which the guinea-pig uterine assay may work to advantage in their own laboratories, whilst others have investigated the "in vitro" assay using the uteri of different animals.

Morell, Allmark and Bachinski[2] described a method using eight small longitudinal strips of uterus, obtained from both uterine horns of a guinea-pig, suspended in Van Dyke and Hastings' solution.[3] The number of strips responding to a dose of pituitary extract was recorded on a smoked kymograph paper. Three doses of the standard solution and three doses of the unknown solution, whose potency was assumed to be equivalent to that of the standard, were administered in various volumes. The larger doses produced responses from between 50 and 87·5 per cent. of the strips. The medium doses produced responses from about 50 per cent., and the smaller doses, responses from about 25 per cent. of the strips. Each dose was given three times in a restricted randomised design. The injections of the standard doses alternated with those of the unknown solution. They observed that it was preferable to give the higher concentrations first. From these quantal responses the potency of the unknown solution could be calculated and a standard error determined. There was no significant difference in the results obtained with uterine strips from the uteri of guinea-pigs in oestrus and in dioestrus. Later, these workers[4] transformed the same technique into a quantitative assay by measuring all responses over 1 mm. produced by the eight uterine strips. The response was related to the logarithm of the dose. Of 23 assays on solutions whose potencies were known, 16 assays produced a range of percentage standard errors that did not include the known potency and thus showed the fallibility of the method.

Hamburger[5] studied the assay of the oxytocic hormone on the isolated uterus of a virgin guinea-pig and used four concentrations of both the standard and the unknown solutions. The ratio between successive concentrations was 2 to 1. Two experimental designs for the injection of doses into the bath were considered, one in which the four concentrations of standard were given in diminishing order, followed likewise by the four doses of the unknown solution. This dose sequence was given three times in all. The second design was to give

doses of the standard alternately with doses of the unknown twice at each of the four dose levels, the latter being randomised.

The interval between doses was 10 minutes. Responses were measured and related to the logarithm of the dose. From these data the potency of the unknown solution could be determined. No complete analysis of the results was given, but Hamburger claimed that a posterior pituitary lobe extract could be standardised with "sufficient accuracy" by the employment of three or four suitable guinea-pig uteri.

Some workers[6,7] have attempted to improve the guinea-pig uterine assay by modifying the magnesium and calcium ion concentrations of the Ringer's solution. They claim thereby to have improved the assay.

Fig. 1 shows the tracing of a test carried out by the assay procedure adopted in our laboratories. The uterus of a virgin guinea-pig was suspended in the physiological salt solution described in the British Pharmacopoeia,[8] maintained at a temperature of 37° C. and aerated with compressed air. Recordings were made on a smoked kymograph paper with a frontal writing lever giving a magnification of × 2. Two different submaximal responses to an extract of the standard were obtained. Next a dose of the unknown solution giving a response intermediate between the two standard responses was found, and the three doses were repeated in the reverse order. The maximum ratio we allow for the two doses of standard is 1·33.

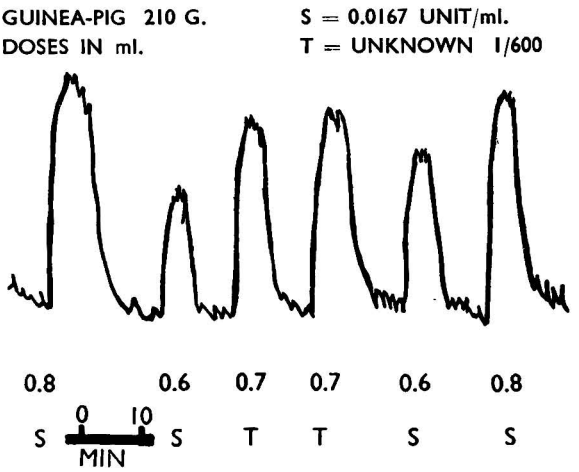

Fig 1.* Typical example of the isolated guinea-pig uterine assay of a posterior pituitary extract.

S = ml. of Extract of the International Posterior Pituitary Standard 2 I.U. per ml.; dilution 1:120.

T = ml. of Unknown; dilution 1:600.

* Fig. 1 and all the tables in this paper are reproduced from the *Journal of Pharmacy and Pharmacology*, 1949, 1, 436–453, by permission of the Editor.

Gaddum[9] studied the precision of the guinea-pig uterine assay when four doses were employed, *e.g.*, standard, unknown, unknown, standard, until the unknown is found to have an activity greater than one particular potency and less than another potency. He discarded results in which the ratio of the two limiting activities was greater than 1·5, and found that the standard error on 43 assays was 7·73 per cent.

Holton[10] described a method employing the isolated uterus of a non-pregnant rat of weight 120 to 200 g., suspended in Locke's solution having one-quarter of the calcium and one-half of the glucose concentrations normally employed. Recordings of the uterine contractions were made with an isotonic and linear lever of Schild's design.[11] The bath was aerated with a mixture of 95 per cent. of oxygen and 5 per cent. of carbon dioxide and kept at a constant temperature of about 32° C. A four-point assay was conducted by the method described by Schild.[12] Doses were administered regularly at intervals of three or four minutes and the mean experimental time of an assay was 3·75 hours, with a mean percentage error of

2·16 on eight satisfactory assays. Fig. 2 is a tracing of such a test. We have confirmed that this is the most precise of the isolated-uterus methods for the assay of the oxytocic hormone.

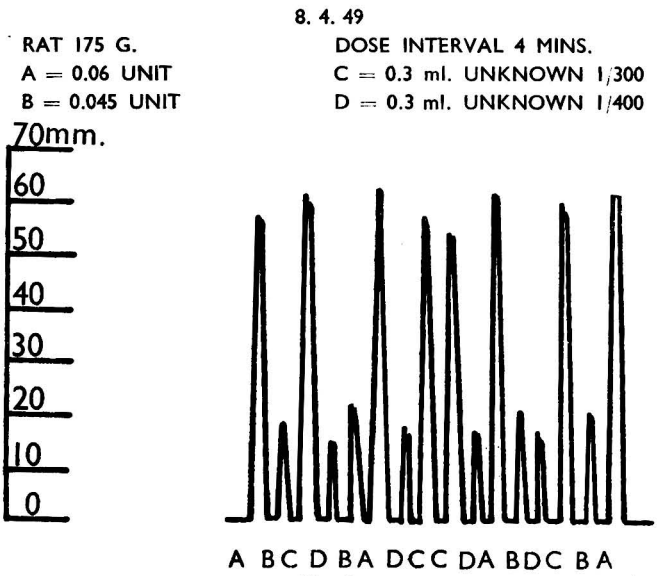

Fig. 2.

Bachinski and Allmark[13] have conducted "in vitro" assays on the uterus of the guinea-pig, rat and rabbit and on strips of human uterine muscle excised during Caesarean sections at or near full term. They found the sensitivity of the rabbit uterine muscle to "Pitocin" and "Pitressin" (Parke Davis preparations of the relatively pure extracts of the oxytocic and vasopressor principles) more closely resembled that of human uterine muscle than did those of the uterine muscles from other animals. A sample of Pitocin assayed against an extract of the Canadian standard posterior pituitary powder gave results on the normal guinea-pig uterus that differed significantly from the assay conducted on the normal rabbit uterus or human uterine muscle. They employed Van Dyke and Hastings' solution[3] throughout their assays, because its magnesium ion concentration approximates to the serum magnesium ion level of man as well as of pregnant woman. It has recently been shown[14] that Pitressin exerts a variable oxytocic activity depending upon the concentration of magnesium and calcium ions in the physiological salt solution: the observations on variations in the concentration of magnesium confirmed the results of Fraser.[15] It was also shown[14] that the sensitivity of guinea-pig uteri to stimulation by Pitocin and Pitressin depends upon the hormonal state of the guinea-pig. Table I shows that the sensitivity of virgin guinea-pig uteri to stimulation by Pitocin

TABLE I

THE SENSITIVITY OF UTERI TO PITOCIN AND PITRESSIN FROM VIRGIN GUINEA-PIGS TREATED WITH PROGESTERONE AND STILBOESTROL

	Uterine sensitivity		Sensitivity ratio, Pitocin/Pitressin
	Pitressin, milliunits/ml.	Pitocin, milliunits/ml.	
Progesterone-treated guinea-pigs	0·04–0·33	0·08–0·26	1·08
Stilboestrol-treated guinea-pigs	0·01–0·04	0·002–0·018	2·50
Sensitivity ratio of progesterone-treated guinea-pigs to stilboestrol-treated guinea-pigs	0·138	0·058	—

is much greater when they have undergone a treatment with stilboestrol than with progesterone. The same was found for the oxytocic effect of Pitressin. The uteri of

stilboestrol-treated guinea-pigs are more sensitive to Pitocin than to Pitressin, whilst the uteri of progesterone-treated guinea-pigs are almost equally sensitive to both.

Because of the complex nature of a pituitary extract and because the ratio of the amount of the oxytocic hormone to the amount of the vasopressor hormone in the extract may not be the same as in an extract of the international standard, exact details of the conditions of assay should be stated when a sample is being compared in different laboratories. Confusion has often arisen over the potency of a particular commercial sample assayed on the guinea-pig uterus in different laboratories.

Table II shows the results of the assay of commercial extracts prepared by six different firms and examined for oxytocic activity under the same conditions on the guinea-pig uterus

TABLE II

RESULTS OBTAINED FOR THE OXYTOCIC ACTIVITY OF COMMERCIAL POSTERIOR PITUITARY EXTRACTS, LABELLED TO CONFORM WITH THE B.P. REGULATIONS OF 10·0 OXYTOCIC UNITS PER ML., WHEN ASSAYED BY THE GUINEA-PIG UTERUS METHOD USING RINGER'S SOLUTION B.P. 1948

Sample	Potency, u./ml.	Limits, u./ml.*
A	10·0	8·6–11·4
B	8·8	7·5–10·0
C	7·8	6·7– 8·9
D	11·0	10·0–12·2
E	8·7	7·5–10·0
F	7·7	6·7– 8·9

* Range of upper and lower doses used in the bracketting arrangement described for the uterine assay.

in the physiological salt solution described in the British Pharmacopoeia.[8] All the ampoules were labelled 10 units per ml. The samples did not possess equal oxytocic activity.

Table III shows the results obtained for the assays of six different mixtures of Pitocin and Pitressin in which the ratio of Pitocin to Pitressin ranged from 4·0 to 0·125. The assays

TABLE III

TYPICAL DIFFERENCES IN GUINEA-PIG UTERINE ASSAY RESULTS USING TWO USUAL FORMULAE FOR THE PHYSIOLOGICAL SALT SOLUTION

Ratio, Pitocin / Pitressin	Actual total oxytocic potency,* u./ml.	Ringer's solution, B.P., 1948, potency found, u./ml.	Ringer's solution, with added magnesium (Hsu, 1948)†
4·00	10·1	8·0	10·6
2·00	10·2	9·2	10·6
1·00	10·4	10·0	10·6
0·50	10·8	9·3	13·6
0·25	11·6	11·2	13·6
0·125	13·2	13·0	19·2

* Allowance has been made for 4 per cent. of oxytocic hormone impurity in Pitressin and for 4 per cent. of vasopressor hormone impurity in Pitocin in computing the actual total oxytocic potencies of the solutions.

† Hsu (1948) Ringer contains 0·045 per cent. of magnesium chloride.

were conducted in two physiological salt solutions, one of which contained the magnesium ion concentration stated in the British Pharmacopoeia[8] and the other a much higher concentration.[7] The assays conducted in the latter solution produced much higher results than those expected when the ratio of Pitocin to Pitressin was 0·5 or less.

Gaddum[16] studied under various conditions the stability of the oxytocic hormone of the British standard of 1926, which later became the 1935 International Standard Powder. We have confirmed his results for powders of the potency required by the United States Pharmacopoeia XIII, *i.e.*, not less than 1000 oxytocic units per gram. In recent years, however, commercial samples have very rarely attained this minimum unitage, and in a comparison of the stability of commercial powders of oxytocic potency less than 1000 units per g. with powders of potency similar to that of the International Standard Powder we obtained different results. Also the ratio of the vasopressor hormone to the oxytocic hormone was not always unity in the less potent powders. McClosky, Miller and Le Messurier[17] have found that in whale hypophysis the ratio of the vasopressor hormone to the oxytocic hormone

is greater than unity. For various reasons, therefore, assays are sometimes attempted in which the ratio of the oxytocic hormone to the vasopressor hormone in the standard differs from that in the sample under test.

Because of these many difficulties with "in vitro" assays we decided to study an "in vivo" assay. Gaddum[18] showed that the depressor effect of posterior pituitary extract on the blood pressure of the fowl was due to the oxytocic hormone. Coon[19] conducted assays on the blood pressure of a cockerel anaesthetised with sodium phenobarbitone. The procedure was to match a response of the standard with an equal response of the unknown solution. Tachyphylaxis gradually develops and thus any method based on matching responses will entail certain inaccuracies.

Blackwell Smith, jun., and Vos, jun.,[20] employing the cockerel, performed, according to the design of Bliss and Marks,[21,22] a four-point assay with a dose injected every 10 minutes in a random arrangement. The latent sources of variation due to the influence of the height of the blood pressure immediately preceding a given response and the size of the preceding dose were ignored. An analysis of the data from several assays by partial regression coefficients showed that small fluctuations in blood pressure and the effect of the preceding dose could be ignored without markedly influencing the accuracy of the assays. Errors up to 18·2 per cent. with an average error of 6·9 per cent. were obtained in their assays of posterior pituitary solutions of known strength.

Thompson,[23] however, pointed out that the main difficulty was to eradicate from experimental comparisons the effect of changing sensitivity and this was only achieved in part by the four-point random dose method, as any change in sensitivity occurring during the time required for the injection of, and response to, the four doses causes variations in the responses, which would contribute to the error of the assay. This can be great in the chicken blood-pressure assay. Thompson therefore used a statistical design similar to that used by Vos[24] for the assay of ergometrine.

Both Coon[19] and Thompson[23] used White Leghorn cockerels. When White Leghorns were in short supply we tried Rhode Island Red cockerels, but the sensitivity of these birds to posterior pituitary extract was extremely small. On two occasions no response was elicited. Light Sussex cockerels appeared to be suitable subjects and we finally adopted them for our assays. The weight range of these birds was from 1·8 to 2·3 kg., and the birds were injected intravenously with 180 mg. of sodium phenobarbitone per kg. We tried other barbiturates but none was as good as sodium phenobarbitone in maintaining a high blood pressure in the bird. The cockerels were prepared for assay according to the description given by Coon. The blood pressure was recorded from the ischiadic artery by means of a capillary mercury manometer, and 8·5 per cent. of sodium citrate was used as anticoagulant. An extract of the International Standard, 2 oxytocic units per ml., was generally diluted to 1 in 10 and occasionally to 1 in 20 with 0·9 per cent. saline. Doses not exceeding 0·4 ml. were injected rapidly into the crural vein regularly every 3, 4 or 5 minutes. The unknown was diluted to a strength assumed to be that of the standard dilution, and assays were carried out on the same bird according to the Schild[12] experimental design and also that described by Vos.[24] If the standard and test materials are similar to each other, the experimental design of Vos[24] gives the more accurate results. Should the standard and unknown materials be dissimilar then by the analysis of variance possible with Schild's experimental design[12] one can say whether the assay is valid or not.

Fig. 3 illustrates the Vos[24] experimental design. The dose of standard is kept constant whilst the three different doses of the unknown are given, so as to produce one response greater than, one equal to, and one less than that produced by the standard dose. We have found that falls in blood pressure of 30 to 60 mm. of mercury give responses linearly related to the logarithms of the doses.

When the blood pressure falls to a low level, ephedrine hydrochloride, 4 to 8 mg. per kg., may be administered to raise it. After this has risen it will remain elevated for a period of 1 to 3 hours and falls only very gradually. If the bird becomes insensitive to posterior pituitary extract then it may be rested for an hour or two, after which the sensitivity has usually returned.

Table IV shows a comparison of the results of six different solutions when assayed by the guinea-pig uterine method, using the physiological salt solution described in the British Pharmacopoeia,[8] and by the chicken blood-pressure method. The column on the extreme right shows the good agreement between the two methods.

TABLE IV

COMPARISON OF THE GUINEA-PIG UTERUS AND CHICKEN BLOOD-PRESSURE METHODS FOR THE MEASUREMENT OF OXYTOCIC ACTIVITY

Sample	Chicken depressor method, u./g. ± S.E. (C)	Guinea-pig uterus, u./g. (G)	Ratio, C/G
A	677 ± 52	700	0·967
B	872 ± 70	875	0·997
C	762 ± 49	637	1·196
D	792 ± 68	700	1·131
E	921 ± 49	900	1·023
F	785 ± 65	824	0·950
G	467 ± 44	500	0·945

Coon,[19] using White Leghorn cockerels, showed that the vasopressor hormone produced a potentiating effect on the depressor response of the bird to oxytocic hormone when the ratio

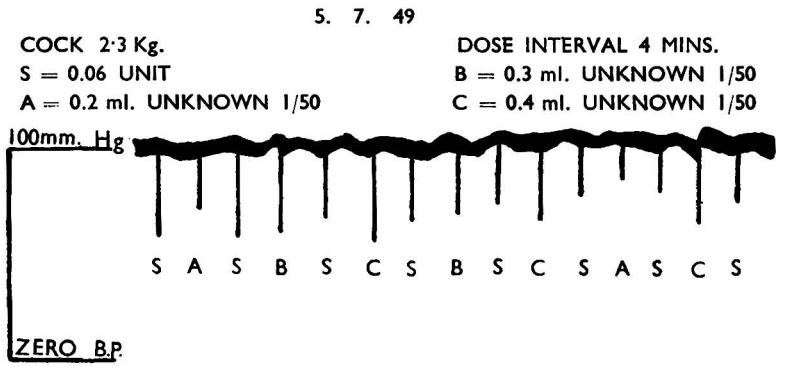

Fig. 3.

of the oxytocic to the vasopressor hormone was less than 0·4. Table V shows the results obtained on the assays of mixtures of Pitocin and Pitressin in Light Sussex cockerels with the

TABLE V

THE INFLUENCE OF DIFFERENT RATIOS OF PITOCIN TO PITRESSIN ON THE ASSAY OF THE OXYTOCIC PRINCIPLE OF THE POSTERIOR PITUITARY LOBE GLAND BY THE CHICKEN DEPRESSOR METHOD

Ratio, Pitocin / Pitressin	Actual total oxytocic potency,* u./ml.	Potency found, u./ml.	Limits of error ($p = 0.95$)
4·00	10·1	10·3	9·3–11·3
2·00	10·2	11·2	8·9–13·5
1·00	10·4	9·7	8·4–11·0
0·50	10·8	10·8	10·0–11·6
0·25	11·6	11·1	10·1–12·1
0·125	13·2	11·1	8·3–13·9
0·100	14·4	18·6	15·0–21·6

* Allowance has been made for 4 per cent. of oxytocic hormone impurity in Pitressin and for 4 per cent. of vasopressor hormone impurity in Pitocin in computing the actual total oxytocic potencies of the solutions

ratio of Pitrocin to Pitressin from 4·00 to 0·100. Only when the ratio of Pitocin to Pitressin is 0·100 or less does the vasopressor hormone produce any appreciable potentiating effect.

The chicken assay is more reliable and more informative in that fiducial limits can be calculated; it requires less skill and experience on the part of the laboratory assistant and less time, 1 to 1·75 hours for an assay involving 10 doses of the unknown solution, compared with 1 to 2 days for the ordinary guinea-pig uterine assay, and it is less expensive than the guinea-pig uterine assay.

THE ASSAY OF THE VASOPRESSOR HORMONE—

Two methods have been widely used for the assay of the vasopressor hormone. Hogben, Schlapp and MacDonald[25] used the spinal cat method. Burn[26] described the preparation of the cat by Elliot's method. The blood pressure was recorded from the left common carotid artery and injections of the drug were made into the right external jugular vein. The blood pressure falls about an hour after the operation and can be maintained at that level for long periods of time. We have found that it is best to leave the preparation at least 2 hours after the operation before beginning the assay. The standard extract of 2 units per ml. is diluted to 1 in 10 with 0·9 per cent. saline and the unknown solution is diluted to a potency assumed to be equal to that of the standard dilution. Injections do not exceed 0·5 ml. Although Burn stated that in some cats the interval between doses can be shortened to half an hour or even 20 minutes, we have found that hourly intervals are better, since this reduced the likelihood of tachyphylaxis. After 10 hours the preparation becomes more sensitive to the vasopressor hormone. Doses used are equal to half what is required to produce maximum response, and responses are matched by the null method. Doses differing by 10 per cent. or more can be distinguished.

Since the time required to assay a sample by the cat method is long, and since there is an acute shortage of experimental cats, we have used the anaesthetised dog in the method described by Kamm, Aldrich, Grote, Rowe and Bugbee.[27] Medium-sized healthy dogs are deeply anaesthetised with an intraperitoneal injection of 0·4 g. of chlorbutol per kg. Cannulas are inserted in a femoral vein and a carotid artery. Injections are made every 15 minutes and doses are kept as small as possible. The method proved to be as accurate as the spinal cat method. Fig. 4 shows the tracings obtained in assays of a solution by the cat and dog methods.

Simon[28] used the effect of the vasopressor hormone on the blood pressure of the decapitated rat and found this to be an extremely sensitive preparation, responding to 2·5 to 5·0 milliunits. Histamine in small doses was without action. Shipley and Tilden[29] have used the pithed rat with satisfactory results.

Stephenson[30] described a preparation of the hind limbs of the rat perfused at room temperature with Ringer - Locke solution through the abdominal aorta. He designed a simple and sensitive outflow recorder for measuring the changes in pressure produced by the pressor hormone. After 4 to 5 hours the preparation is said to respond to 2 milliunits or even 1 milliunit of posterior pituitary extract. Some preparations are known to last 24 hours. In our experience it was found after a few experiments that the preparation failed to recover after the second or third injection of the drug into the perfusate.

THE ASSAY OF THE ANTIDIURETIC HORMONE—

Although most types of animals have been used at one time or another for assay of the antidiuretic hormone, the method described by Burn[31] has been widely employed. Sixteen male rats, weighing 140 to 240 g., are divided into two groups. Each rat is given 5 ml. of water per 100 g. of body-weight by stomach tube. The posterior pituitary extracts, standard and unknown, are injected subcutaneously into the two groups of rats respectively, and the urine excreted is collected. The assay is based upon the time interval between the administration of water and the maximum excretion of urine during periods of 15 minutes. The time elapsing is greater the larger the dose of the extract. The potency is calculated from a previously determined curve relating dose to time elapsing before the maximal rate of excretion occurs. The estimated results, according to Burn, differed from the true results as much as 23 per cent. and the average variation was 12 per cent.

Recently, Theobald, Graham, Campbell, Gange and Driscoll[32] have made reference to assays of the antidiuretic hormone by the method described by Verney and his school.[33,34] Theobald has shown that the intravenous injection of from 0·5 to 10 milliunits of "Infundin" (Burroughs Wellcome preparation) inhibited water diuresis in the dog and man and in woman during the last few weeks of pregnancy. The amount of antidiuretic activity necessary to inhibit water diuresis was remarkably constant for each dog, and the water-diuresis curves obtained over a period of several months could be almost superimposed. He adds that the antidiuretic activity affords the most delicate and possibly the most precise method of assaying posterior pituitary extracts, and he assumes that the vasopressor hormone and the antidiuretic hormone are one and the same principle.

Heller,[35,36] however, has prepared from the vasopressor fraction an extract that contains a high proportion of antidiuretic activity and very little vasopressor activity.

Wokes[37] found in a series of assays of commercial posterior pituitary lobe preparations, assayed for oxytocic potency on the guinea-pig uterus and for antidiuretic potency on the rat, that four out of 20 assays gave results showing a disproportion of the two active principles that he claimed was significant. We have found disproportionate amounts of the oxytocic and vasopressor hormones in commercial pituitary extracts.

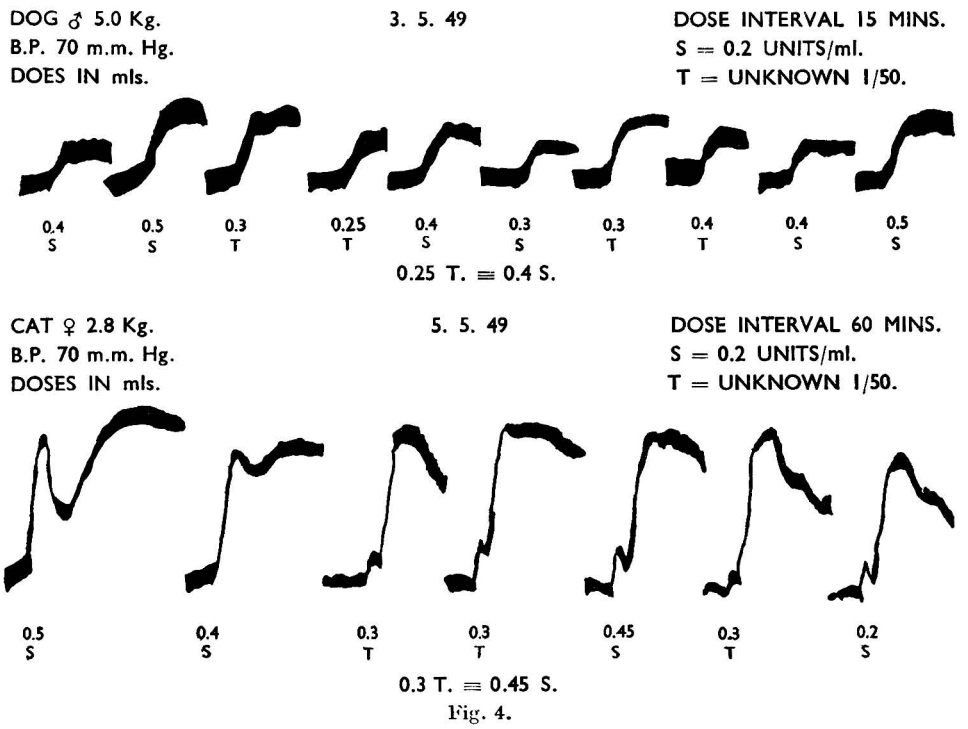

Fig. 4.

When the potency of one of the posterior pituitary hormones in a pituitary extract is required, a method should be employed that measures only that active principle. It should not be assumed, for example, that if the extract contains 10 vasopressor units per ml. when assayed against the International Standard, it will necessarily contain an equivalent unitage of the oxytocic and antidiuretic principles.

I wish to thank the Directors of the Wellcome Foundation Ltd. for permission to publish this paper.

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WELLCOME BIOLOGICAL CONTROL LABORATORIES
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DISCUSSION

PROFESSOR GADDUM enquired whether it was possible to obtain preparations of human uterus that were more sensitive to the vasopressor than to the oxytocic hormone.

MISS MACAULAY enlarged on Professor Gaddum's mention of the anaesthetised rat method[1] for assaying the vasopressor activity of the posterior lobe of the pituitary. In their experience the chief advantages of the preparation were (*a*) its sensitivity—it responded to 0·001 i.u. of vasopressor activity, while 0·005 i.u. gave a rise in blood pressure of 30 to 40 mm.; (*b*) it did not respond to the oxytocic fraction; (*c*) 10 per cent. discrimination was easily obtained; (*d*) no tolerance developed to injections made every 10 minutes—usually 30 to 40 injections were made into the same animal; (*e*) it was completely insensitive to the histamine concentrations encountered in the worst commercial powders.

DR. F. J. DYER asked the author if he would favour making routine assays on low potency commercial pituitary powders by *two* methods, oxytocic and vasopressor (or antidiuretic) and expressing the answer as a ratio of the one to the other. The International Standard was assumed (by B.P. definition) to possess an oxytocic to vasopressor ratio of 1 to 1.

MR. STEWART said that Russell[2] had claimed that Pitressin had a much greater oxytocic effect than Pitocin on human uterine strips excised at term. More recently, Bachinski and Allmark conducted assays on a statistical basis and found that Pitocin and Pitressin were equally active in oxytocic potency on an isolated uterine strip taken at the peak of pregnancy. He himself had not conducted assays with this type of preparation.

Replying to Dr. Dyer, Mr. Stewart said that the use of the rabbit uterine assay and the expression of the answer in terms of one hormone only was possibly the most suitable method. At least Bachinski and Allmark had shown that assays conducted on the normal rabbit and parturient human uteri were comparable. However, the best method could only be stated after further clinical researches had been made into the mode of action of these hormones.

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Direct Electrolysis of Copper from Silver Chloride Filtrates

By GEORGE NORWITZ

Synopsis—Copper can be deposited directly from silver chloride filtrates if an excess of hydrogen peroxide is present. The procedure saves 3 hours in the analysis of alloys containing silver and copper, as it avoids the necessity for evaporating the sulphuric acid until it fumes to remove the chloride ion.

In the analysis of alloys containing silver and copper, it is the usual practice to dissolve the sample in nitric acid, determine silver as silver chloride, and determine copper electrolytically in the silver chloride filtrate. Before depositing the copper, however, it is necessary to evaporate the sulphuric acid until it fumes[1] to remove the hydrochloric acid, since hydrochloric acid interferes with the electrolytic determination of copper.[3] This evaporation takes about 3 hours, and obviously increases the chances of error. The author has found that by adding an excess of hydrogen peroxide to the silver chloride filtrate, the copper can be deposited directly in the presence of the hydrochloric acid.

Hydrazine sulphate has been used for the electrolytic determination of copper in the presence of hydrochloric acid.[2] Attempts by the author, however, to use this reagent for the electrolytic determination of copper in silver solders failed. This failure can be traced to the fact that hydrazine sulphate cannot be used in the presence of more than very small amounts of nitric acid.[2]

Experimental

A silver nitrate solution was prepared by dissolving 15·7 g. of silver nitrate in water and diluting to 1 litre. Ten millilitres of this solution contained approximately 0·1 g. of silver.

A standard copper nitrate solution was prepared as follows: 5·000 g. of pure electrolytic copper were dissolved in 40 ml. of diluted nitric acid (1 + 1) by warming on a steam bath. The oxides of nitrogen were boiled off and the solution was cooled and diluted to 500 ml. in a volumetric flask.

Varying amounts of silver nitrate solution and standard copper nitrate solution were measured into 300-ml. beakers. Measuring cylinders were used to measure the silver nitrate solution, and pipettes for the copper nitrate solution. The solutions were diluted to 150 to 175 ml. with water, and were treated with 3 ml. of 70 per cent. nitric acid. The silver was precipitated as the chloride according to the A.S.T.M. method,[1] and the solutions filtered through Gooch filters. Four millilitres of 70 per cent. nitric acid and 30 ml. of 3 per cent. hydrogen peroxide were added to the filtrates, and the copper was electrolysed at 2 amperes for 1 hour with platinum gauze cathodes, 60 mm. in height and 50 mm. in diameter, and platinum spiral anodes. During the electrolysis the solutions were stirred. The results obtained for copper, as can be seen from Table I, were excellent. The deposits were invariably bright and adherent.

The above experiment was repeated on two samples without the addition of the hydrogen peroxide. The copper plated out was so dark that the electrolysis was discontinued after 10 minutes for fear of injuring the platinum.

Table I

Results for copper in synthetic mixtures of copper and silver

Silver present, g.	Copper present, g.	Copper found, g.
0·25	0·7500	0·7499
0·25	0·7500	0·7496
0·50	0·5000	0·4996
0·50	0·5000	0·5001
0·75	0·2500	0·2498
0·75	0·2500	0·2498
0·10	0·1000	0·0997
0·10	0·1000	0·1001

A typical silver solder was analysed by the A.S.T.M. procedure,[1] and found to contain 15·47 per cent. of copper, 49·90 per cent. of silver, 17·97 per cent. of cadmium and 16·60 per cent. of zinc. This sample was analysed for copper four times by the following modification of the method described here. A 1-g. sample was dissolved in 20 ml. of diluted nitric acid (1 + 4), and the silver precipitated as the chloride according to the A.S.T.M. procedure.[1] The silver chloride filtrate was treated with 4 ml. of 70 per cent. nitric acid and 30 ml. of 3 per cent. hydrogen peroxide, and electrolysed for copper. The results obtained for copper are shown in Table II. The method shows high accuracy and precision.

TABLE II

RESULTS FOR COPPER IN A TYPICAL SILVER SOLDER

Copper present, %	Copper found, %
15·47	15·52
	15·47
	15·50
	15·48
	Average 15·49 + 0·02

To find out how much hydrochloric acid could be present during the electrolysis the following experiment was performed. Six 25-ml. portions of standard copper solution were pipetted into 300-ml. beakers, and 100 ml. of water and 7 ml. of 70 per cent. nitric acid were added to each portion. Amounts of 36 per cent. hydrochloric acid ranging from 0 to 5 drops were added, followed by 30 ml. of 3 per cent. hydrogen peroxide. The solutions were diluted to 200 ml. and electrolysed for copper. The results obtained for copper, as shown in Table III, were satisfactory, but the deposits obtained in the presence of 4 to 5 drops of hydrochloric acid were slightly discoloured. No attempt was made to use more than 5 drops of hydrochloric acid.

TABLE III

RESULTS FOR COPPER USING VARIOUS AMOUNTS OF HYDROCHLORIC ACID

Hydrochloric acid present, drops ..	0	1	2	3	4	5
Copper found, g.	0·2498	0·2501	0·2496	0·2495	0·2499	0·2496

Two platinum anodes that had been used throughout the experiments with solutions containing hydrogen peroxide were weighed before and after the series of electrolyses. One was found to have lost 0·3 mg. after eight runs and the other 0·2 mg. after ten runs. The average loss was 0·028 mg. per run. This is approximately the usual loss obtained in electrolysing copper from nitric acid solutions which do not contain hydrochloric acid.

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Separation of Thorium from the Ceria Earths and its Application to the Analysis of Monazite

By M. VENKATARAMANIAH and BH. S. V. RAGHAVA RAO

Synopsis—Thoria can be separated from the ceria earths in ratios up to 1 : 16 by a single precipitation with sodium naphthionate in nearly neutral solution. The minimum amount of thoria that can be satisfactorily determined is about 9 mg. The method can be used for the determination of thoria in the rare earths obtained from monazite.

The importance of thorium in fission studies has stimulated interest in the chemistry of the element and in particular in its analytical reactions. Monazite still remains the chief source of thorium and the separation of the element from the associated cerite earths is a problem of some complexity.[1] With the exception of the iodate method of Meyer and Speter,[2] which is both cumbersome and expensive, all reported procedures require preliminary removal of zirconia and phosphoric acid. In addition, double and sometimes triple precipitations are necessary for the effective removal of ceria earths. Whilst retaining the former disadvantages the procedure described in this paper offers a simple but accurate method of separating thoria by a single precipitation.

Procedure—

Make the solution, containing not more than 0·2 g. of thoria, just acid to Congo red. The exact acidity is important, because if the solution is more acid, precipitation of thoria is not complete, and if the acidity is lower, the precipitate is flocculent and retains large amounts of the mother liquor, making later washing difficult and even ineffective. These qualitative directions are sufficient to carry out a separation. It has, however, been found that if the pH of the solution is lower than 2·3, precipitation of thoria is incomplete, and contamination with ceria begins at a pH of 3·2.

Dilute the solution to 100 ml. and boil. To the boiling solution add slowly and with constant stirring 100 ml. of a boiling 10 per cent. solution of sodium naphthionate. A pink crystalline precipitate forms at once. Continue boiling for 5 to 10 minutes and set aside to cool. Filter the cold liquid through a 9-cm. Whatman No. 42 filter-paper and wash the precipitate three times by decantation with cold water and finally four or five times on the filter-paper. Ignite wet, and weigh as ThO_2.

To determine the ceria earths, add ammonia to the filtrate and washings until the solution is alkaline. Filter, wash, ignite and weigh the precipitate as ceria earths; a correction for excess oxygen over R_2O_3 is made by dissolving in hydrochloric acid and potassium iodide and titrating the liberated iodine with sodium thiosulphate.

Results—

Table I gives the results obtained by this method with thoria and artificial mixtures of thoria and ceria earths.

Determination of thorium in monazite—

The mixture of thoria and rare earth oxides obtained from a 30-g. sample of monazite from Travancore, India, according to the procedure prescribed by Miner,[3] and freed effectively from phosphoric acid by double precipitation with oxalic acid, is dissolved in concentrated nitric acid and a few ml. of hydrogen peroxide to reduce Ce^{IV} to Ce^{III}, and the solution is evaporated almost to dryness on a water-bath. The residue is dissolved in water and the solution made up to 500 ml. In 25 ml. of this solution the thoria and the ceria earths are determined as described above. The results of four analyses made in this way together with two by Neish's *m*-nitrobenzoic acid method[4] (double precipitation) are shown in Table II.

TABLE I

APPLICATION OF PROPOSED METHOD TO ARTIFICAL MIXTURES

ThO_2 taken, g.	R_2O_3 added, g.	ThO_2 found, g.
0·0087	nil	0·0088
0·0087	nil	0·0089
0·0087	0·0866	0·0088
0·0087	0·0866	0·0087
0·0438	0·0866	0·0440
0·0438	0·0866	0·0437
0·0875	nil	0·0878
0·0875	nil	0·0876
0·0875	0·0700	0·0873
0·0875	0·0700	0·0874
0·0875	0·2915	0·0874
0·0875	0·2915	0·0876
0·0875	0·5830	0·0876
0·0875	0·5830	0·0877
0·0875	0·8745	0·0875
0·0875	0·8745	0·0877
0·0875	1·4575	0·0873
0·0875	1·4575	0·0875
0·1750	1·4575	0·1752
0·1750	1·4575	0·1748

TABLE II

Sodium naphthionate method		Neish's *m*-nitrobenzoic acid method	
ThO_2, g.	R_2O_3, g.	ThO_2, g.	R_2O_3, g.
0·1314	0·7595	0·1315	0·7596
0·1317	0·7594	0·1318	0·7592
0·1312	0·7596		
0·1316	0·7592		

SUMMARY AND CONCLUSIONS

In neutral nitrate solution sodium naphthionate under proper conditions effects in a single precipitation complete separation of thoria from up to sixteen times the weight of ceria earths. As little as 9 mg. of thoria can be satisfactorily determined. The method has been successfully applied to the determination of thoria in monazite. The reagent offers a distinct advantage over others so far suggested.

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The Use of Tannin in the Estimation of Zirconium and its Separation from Thorium

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Synopsis—Zirconium is quantitatively precipitated by tannin from a chloride solution up to 0·15 *N* in hydrochloric acid. By double precipitation at this acidity it can be quantitatively separated from thorium.

Schoeller[1] put forward a claim for tannin as a selective reagent for zirconium, but his results in its separation from thorium are not quite convincing in that his total zirconium dioxide values are high and even in the one instance of close agreement the zirconium dioxide precipitate contained some thorium. A careful perusal of Schoeller's results showed that a fuller study of the precipitation of zirconium under various conditions might throw further light on the problem and yield fruitful results.

With this object, the effect of acidity on the precipitation of zirconium by tannin was studied and the results were utilised to devise a procedure for its separation from thorium.

Experimental

Determination of zirconium—

From a stock solution of pure zirconyl chloride in 0·1 *N* hydrochloric acid, 20-ml. portions were taken and the zirconium content was determined by precipitation with mandelic acid[2] and with *m*-nitrobenzoic acid.[3] The mean weights of ZrO_2 obtained were 0·0550 g. and 0·0552 g. respectively. From the weighted mean of all the results the ZrO_2 content of the solution was taken as 0·0550 g. per 20 ml. Aliquots (20 ml.) of this solution were neutralised by careful addition of calculated quantity of ammonia or further acidified with 2 *N* hydrochloric acid to bring the acidity to the desired value when diluted to 200 ml.; 10 ml. of saturated ammonium chloride solution were added and the solution was diluted to 175 ml. and boiled. A freshly prepared solution of 1 g. of tannin in 25 ml. of water was then added, and the resulting precipitate was allowed to settle, filtered, washed with a 2 per cent. solution of ammonium chloride and ignited to ZrO_2, following the directions given by Schoeller. The results obtained are given in Table I.

Table I

Effect of acidity on precipitation of zirconium dioxide

Conditions of precipitation	Weight of ZrO_2, g.
Just neutral to Congo red	0·0553
	0·0552
	0·0550
	0·0549
0·1 *N* hydrochloric acid	0·0550
	0·0553
0·15 *N* hydrochloric acid	0·0550
	0·0549
	0·0548
	0·0549
0·2 *N* hydrochloric acid	0·0541
	0·0539

These results show that precipitation of zirconium is complete in solutions up to 0·15 *N* in hydrochloric acid; at higher acidity, part of the metal remains in solution. In confirmation, when the filtrate from the two experiments in 0·2 *N* hydrochloric acid was neutralised, a slight turbidity resulted.

SEPARATION OF ZIRCONIUM FROM THORIUM—

A semi-quantitative test revealed that in 0·15 *N* hydrochloric acid there was no immediate precipitation of thorium and only a slight turbidity occurred on boiling for half an hour. If zirconium is present, however, fair quantities of thorium may be co-precipitated. In the following experiments, to the combined zirconium and thorium solutions, 1·5 g. of tannin (as against 1 g. previously) in 25 ml. were added, and the solution was boiled for 2 minutes after addition of the reagent. The precipitate was allowed to settle for 2 hours, filtered through a Whatman No. 42 filter-paper, washed and ignited to ZrO_2. The results are given in Table II.

TABLE II

PRECIPITATION OF ZIRCONIUM DIOXIDE IN PRESENCE OF THORIUM

ZrO_2 taken = 0·0550 g.; acidity = 0·15 *N*

Expt. No.	ThO_2 added, g.	ZrO_2 weighed, g.
1	0·1740	0·0623
2	0·1740	0·0621
3	0·0870	0·0594
4	0·0870	0·0594

In all these experiments the filtrate was free from zirconium. The filtrate in experiment 1 was evaporated to dryness and the residue ignited and fused with potassium pyrosulphate, the melt extracted with water, and the solution tested qualitatively with sodium hydrogen phosphate in 10 per cent. sulphuric acid. No trace of zirconium was detected. Complete separation of thorium was obtained by redissolving the first tannin precipitate and reprecipitating the zirconium as described below.

PROCEDURE—

Treat the zirconium - thorium chloride solution, which must be free from sulphate, with 20 ml. of saturated ammonium chloride solution and neutralise to Congo red with ammonia. Add the calculated quantity of 2 *N* hydrochloric acid to give a solution which is 0·15 *N* with respect to the acid when the volume is made up to 200 ml. Heat the solution to boiling, add 1·5 g. of tannin, freshly dissolved in 25 ml. of water, gently boil for a minute, and set aside to settle for 2 hours. Filter through a Whatman No. 42 filter-paper, wash the precipitate on the filter three times with 10 to 15-ml. portions of a solution of 10 per cent. of ammonium chloride in 0·15 *N* hydrochloric acid. Transfer the precipitate and the filter-paper to the original beaker, add 60 ml. of diluted hydrochloric acid (1 + 1) and boil gently for 15 to 20 minutes. Set aside in a warm place overnight. With Congo red as indicator, carefully neutralise the excess acid with diluted ammonia solution (1 + 6) and add 20 ml. of ammonium chloride solution and sufficient 2 *N* hydrochloric acid to make the acidity once again 0·15 *N* when the solution is diluted to 300 ml. Dilute, boil and add 1·5 g. of freshly prepared tannin solution. Continue boiling for another 2 minutes and set aside for 2 hours. Filter through a Whatman No. 42 filter-paper and wash the precipitate first with a solution of 10 per cent. of ammonium chloride in 0·15 *N* hydrochloric acid and finally with a 2 per cent. solution of ammonium chloride alone. Ignite and weigh as ZrO_2. To determine the thorium, concentrate the combined filtrates to a small volume, add a slight excess of ammonia followed by a further 1 g. of tannin and heat to boiling. Allow the precipitate to settle, filter, and wash it with a solution of 2 per cent. of ammonium chloride; ignite and weigh as ThO_2. The results of test separations are given in Table III.

TABLE III

TEST SEPARATIONS

ZrO_2 taken = 0·0550 g.

Expt. No.	ThO_2 added, g.	ZrO_2 weighed, g.	ThO_2 found, g.
1	0·0870	0·0550	0·0860
2	0·0870	0·0547	—
3	0·0870	0·0545	—
4	0·0870	0·0549	—
5	0·1740	0·0548	0·1745
6	0·1740	0·0551	—

The residual zirconium dioxide from experiments 1 and 6 was fused with potassium bisulphate and the melt extracted with 0·5 *N* hydrochloric acid. The extract was boiled with saturated oxalic acid solution. No turbidity appeared even after standing overnight. This shows that the zirconium precipitate was free from thorium.

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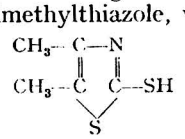
The Colorimetric Determination of Rhodium with 2-Mercapto-4:5-Dimethylthiazole

BY D. E. RYAN

SYNOPSIS—Rhodium is determined by the formation of the rhodium complex $(C_4H_6NSCS)_2Rh$, with 2-mercapto-4 : 5-dimethylthiazole and photometric measurement of the optical density of the resulting coloured solution. Platinum and gold do not interfere; interference by palladium is avoided by addition of dimethylglyoxime and filtration. Iridium, in an amount equivalent to that of rhodium, gives results 10 per cent. high; iridium is readily compensated for, however, if its approximate concentration is known.

VARIOUS organic monosulphides have been suggested for the determination of rhodium.[1,2,3,4] In all of these methods other platinum metals cause varying degrees of interference. The colorimetric determination of rhodium with 2-mercaptobenzoxazole,[2] while providing a method for determining rhodium in the presence of iridium, requires precipitation to effect the desired colour formation, and also suffers interference from platinum and palladium. In this paper is described a procedure for determining 0·5 to 8 μg. of rhodium per ml. in the presence of platinum, palladium, iridium and gold. The determination is based on the colour produced with 2-mercapto-4 : 5-dimethylthiazole, which has the formula



The possibility of using 2-mercapto-4 : 5-dimethylthiazole as a colorimetric reagent for rhodium was suggested previously by Haines and Ryan.[3] Acid solutions of rhodium develop an amber-to-red colour on being heated with 2-mercapto-4 : 5-dimethylthiazole and, when there is an excess of reagent present, the intensity of the colour depends upon the amount of rhodium.

EXPERIMENTAL

The transmittancy curve for a solution containing 6·5 μg. of rhodium per ml. is shown in Fig. 1. The data were obtained with a Spekker absorptiometer used in conjunction with a medium glass spectrograph (constant deviation instrument). All matching on the plate was done visually and monochromatic light from a mercury arc was used for determining wavelengths. Although maximum absorption occurs in the ultra-violet, excellent results can be obtained in the visible spectrum.

A Spekker photo-electric absorptiometer, with an Ilford No. 601 filter (maximum transmittancy at 430 mμ.), was used to obtain the data for the development of the colorimetric method for rhodium.

The maximum concentration that could be measured in a 1·00-cm. cell was found to be 8 μg. of rhodium per ml., while a concentration of 0·5 μg. per ml. gave a minimum transmittancy of 88 per cent. More or less highly concentrated solutions can be measured when very thin or very thick layers of solution are used.

REAGENTS—

2-Mercapto-4 : 5-dimethylthiazole—The reagent was obtained from the Eastman Kodak Company and was recrystallised from 25 per cent. ethyl alcohol. A solution was prepared by dissolving 0·5 g. of the recrystallised material in 100 ml. of 50 per cent. ethyl alcohol. This solution was colourless and was stable for several months if kept in a dark bottle; solutions kept in colourless bottles turned yellow within two weeks. 2-Mercapto-4 : 5-dimethylthiazole can be prepared by the method of Buchman, Riems and Sargent.[5]

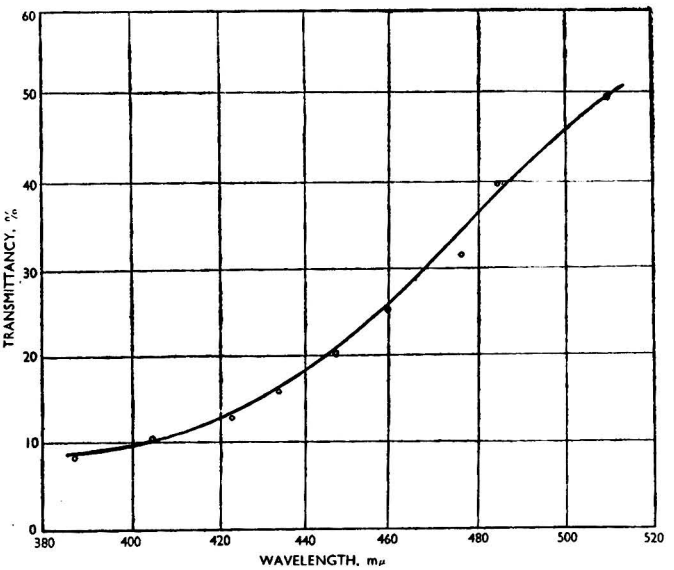


Fig. 1. Transmittancy curve for solution containing 6·5 μg. of rhodium per ml.

Standard rhodium solution—Rhodium sponge was converted into the chloride by means of concentrated hydrochloric acid and chlorine gas. The resulting solution was evaporated just to dryness and the residue dissolved in a solution containing 1 ml. of concentrated hydrochloric acid per litre. This solution, standardised by both the sulphide[6] and mercaptobenzoxazole[3] procedures, contained 1·32 mg. of rhodium per ml. Suitable concentrations were prepared by diluting this standard with a solution containing 1 ml. of concentrated hydrochloric acid per litre.

PROCEDURE—

Measure rhodium samples, either as the chloride or sulphate, into Erlenmeyer flasks, add 10 ml. of concentrated hydrochloric acid, and make the volume up to about 40 ml. Heat the solutions to boiling, add 2 ml. of reagent for each microgram of rhodium per ml. expected, and boil the solutions vigorously for 1 hour. Keep the volume approximately constant by adding distilled water. Cool the samples in running water, make up the volume to 100 ml., and measure the optical density with the absorptiometer.

The optical densities obtained under various conditions are shown in Table I. These results were obtained with 0·7 μg. of rhodium per ml. using a 4-cm. cell.

TABLE I

OPTICAL DENSITY OF SOLUTIONS OF 0·7 μG. OF RHODIUM UNDER VARIOUS CONDITIONS

Condition	Optical density
Solutions boiled 45 minutes	0·306
Solutions boiled 60 minutes	0·319
Solutions boiled 120 minutes	0·318
Reagent added, 1·0 ml.	0·292
Reagent added, 1·5 ml.	0·320
Reagent added, 2·0 ml.	0·319
Concentrated HCl added, 4 ml.	0·293
Concentrated HCl added, 8 ml.	0·318
Concentrated HCl added, 15 ml.	0·319

These data indicate that 2 ml. of 0·5 per cent. reagent solution for each microgram of rhodium per ml. and boiling for 1 hour are necessary for maximum colour development. The optical densities of solutions ranging from 1 to 2 *M* in hydrochloric acid were the same and no difficulty was encountered in maintaining clear solutions.

Extinction - concentration graphs were plotted and showed that the coloured solution obeys Beer's law over the range applicable for a 1·00-cm. cell. The change in optical density on keeping the solutions in daylight in a closed container for 48 hours was less than 3 per cent.

Composition of complex

The rhodium complex with 2-mercapto-4 : 5-dimethylthiazole was precipitated from a solution 0·1 *M* in hydrochloric acid. The complex could not be dried at elevated temperatures because it decomposed. The average value obtained for the rhodium content, on analysis of the complex dried in a desiccator over silica gel, was 27·0 per cent. The theoretical value, if two molecules of 2-mercapto-4 : 5-dimethylthiazole are combined with one atom of rhodium, is 26·4 per cent.

Effect of various anions

No interference was noted when 0·5 g. of the sodium, potassium or ammonium salts of the following ions was added to 0·066 mg. of rhodium, as the chloride, and the usual colorimetric procedure carried out: F', Cl', Br', C_2O_4'', $C_6H_7O_7'$, SO_4'', $C_2H_3O_2'$, PO_4''', $C_4H_4O_6'$.

Nitrate, chlorate and bromate interfere if more than 50 μg. per ml. are present. These oxidising agents prevent the reduction of Rh''' to Rh'' by the reagent unless a sufficient excess of the latter is present. Complete colour development is possible in solutions containing 500 μg. of these ions per ml. if a suitable excess of reagent is added.

Carbonate and cyanide interfere if more than 40 μg. per ml. are present. Iodide and sulphite ions interfere by producing turbid solutions.

Reactions of various cations

Studies of the reaction of various ions under conditions similar to those used for rhodium —in which 5 mg. of the metal ions, as chloride, sulphate, or acetate, were used—showed no colour or precipitate with the following: Fe''', Co'', Ni'', Pb'', Zn'', Sn'', Sn'''', Al''', Sb''', Ba'', Be'', Ca'', Mg'', Mn'', Ti'''', UO_2'', Cd'', K' and Na'. Furthermore, about 5 mg. of tungsten (as sodium tungstate), molybdenum (as ammonium molybdate) and arsenic (as potassium arsenate) gave no noticeable reaction.

The cupric ion gave a yellow precipitate and bismuth gave a solution coloured a faint yellow. The mercuric ion gave no reaction under the acid conditions used for the rhodium determination, but reacted to give a white precipitate in neutral medium. Silver gave a yellow precipitate on addition of the reagent to an aqueous solution of silver nitrate. Chromate was reduced to tervalent chromium, the solution becoming pale green in colour.

Of particular interest, however, are the reactions of gold, platinum, palladium and iridium, as these elements are usually associated with rhodium. Gold ($AuCl_4'$) solutions gave an immediate white precipitate. Gold, in solutions treated in the same manner as for rhodium, was completely precipitated. Filtrates from solutions containing 15 μg. of gold per ml. were colourless and showed no difference from the blank when checked in the absorptiometer.

Platinum ($PtCl_6''$) solutions reacted to give a yellow solution or precipitate depending on the platinum concentration, but platinum, in solutions treated in the same manner as for rhodium, was completely precipitated. Filtrates from solutions containing 3 μg. of platinum per ml. were colourless and showed no difference from the blank when checked in the absorptiometer.

Palladium ($PdCl_4''$) solutions gave an amber-to-red colour similar to that given by rhodium. Solutions containing even as little as 0·3 μg. of palladium per ml. gave a colour noticeably different from the blank; palladium must therefore be removed before determining rhodium.

The very dark red iridium ($IrCl_6''$) solutions were changed in colour to a very pale olive-green. Solutions treated in the same manner as for rhodium, however, developed a more intense greenish-yellow colour and 3·5 μg. of iridium per ml. gave an optical density equivalent to 0·4 μg. of rhodium per ml.

The determination of rhodium when other platinum metals are present

Platinum and gold—

Since platinum and gold are precipitated by 2-mercapto-4 : 5-dimethylthiazole to yield colourless filtrates, the following procedure allows the direct determination of rhodium in the presence of these elements.

Procedure—Treat solutions as previously described for rhodium but add 2 ml. of reagent in excess of that necessary for rhodium. Cool the solutions in running water for 20 minutes, dilute to about 70 ml. and filter off the precipitates. Wash the precipitates once with 5 ml. of cold water, make up the filtrate to 100 ml., and measure the optical density. Typical results are shown in Table II.

Table II

Determination of rhodium in presence of iridium, palladium, platinum or gold

Rh present, μg. per ml.	Rh found, μg. per ml.	Other metal present, μg. per ml.	Remarks
0·7	0·7	0·7 Ir	Ir not allowed for in blank
0·7	0·8	1·3 Ir	"
3·3	3·7	3·5 Ir	"
6·6	7·3	6·0 Ir	"
1·3	1·3	3·5 Ir	Ir compensated in blank
6·6	6·6	6·0 Ir	"
0·7	0·7	5·0 Pd	Pd first separated by dimethylglyoxime
3·3	3·4	8·4 Pd	"
6·6	6·7	17·0 Pd*	"
6·6	6·5	34·0 Pd	"
6·6	8·4	3·0 Pd	Pd not separated
0·7	0·7	3·0 Pt	Pt precipitated by reagent and filtered
0·7	0·7	6·0 Pt	"
6·6	6·6	3·0 Pt	"
6·6	6·5	12·0 Pt	"
6·6	6·6	15·0 Pt*	"
6·6	6·5	17·0 Au	Au precipitated by reagent and filtered
6·6	6·6	15·0 Au*	"
6·6	6·6	34·0 Au	"

* These results were obtained independently by a member of this laboratory. The blank, except where stated for iridium, was distilled water.

Palladium—

Palladium must be removed before determining rhodium. This is accomplished by precipitating the palladium with dimethylglyoxime and determining rhodium directly in the filtrate from the palladium determination.

Procedure—Add 1 ml. of a 1 per cent. alcoholic solution of dimethylglyoxime to 50 ml. of a solution containing approximately 0·7 ml. of concentrated hydrochloric acid. Allow the solution to stand for 1 hour and filter through a porcelain crucible with a porous bottom. Wash the precipitate three times with 5-ml. portions of hot water, add 10 ml. of concentrated hydrochloric acid to the filtrate, and carry out the regular procedure for rhodium. Typical results are shown in Table II.

Iridium—

Iridium, when present in an amount equivalent to that of rhodium, gives results that are about 10 per cent. high. When 3·3 μg. of rhodium per ml. was determined in the presence of 3·5 μg. of iridium per ml., the rhodium found was 3·7 μg. If the amount of iridium present is known, however, its interference can readily be compensated for. Some results are shown in Table II.

The author thanks Mr. L. S. Theobald, in whose laboratory this research was carried out, for his encouragement and help in this work. Thanks are also due to W. P. Doyle for his help in obtaining the transmittancy data, and to Miss M. E. Dalziel for determining rhodium in various platinum metal solutions by the dimethylthiazole procedure.

The author wishes to record his sincere appreciation to Lord Beaverbrook for providing the scholarship, through the Beaverbrook Overseas Scholarship Fund, which enabled this work to be completed.

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Notes

THE MICRO-KJELDAHL DETERMINATION OF SERUM PROTEINS, WITH SELENIUM AND COPPER AS CATALYSTS: INTERRELATION OF TIME OF DIGESTION AND CONSTITUENTS OF THE DIGESTION MIXTURE

In the micro-Kjeldahl determination of protein, a consideration of such factors as the digestion time and the amount of salt and catalyst shows that they are not independent. We have investigated the interrelationship of these factors as applied particularly to serum proteins by using a combination of selenium dioxide and copper sulphate as catalysts, and have also determined the optimum conditions for the complete recovery of nitrogen.

In all the experiments, 0·1 ml. of serum was used with 1 ml. of sulphuric acid, the copper sulphate being added as a saturated solution and the selenium dioxide (in aqueous solution) introduced after the contents of the flask were charred. Heating during the digestion was carried out in such a way that the acid vapours just condensed in the lower part of the neck of the flask, the upper part being barely warm to the touch. Experiments have shown that unless these conditions of heating are carefully adhered to the recovery of nitrogen will be low. The ammonia distillation apparatus was similar to that of Markham.[1] The distillate was received in boric acid solution and titrated with standard sulphuric acid using Tashiro's indicator.

As the nitrogen content of the serum used was unknown, the analyses were invariably made in replicate (never less than in duplicate). The results were analysed statistically. All of the mean results that fell within four times the standard error, that is within $4 \times 0{\cdot}01/\sqrt{2}$ of the maximum mean titre were taken as indicating complete recovery. The term 0·01 represents the standard deviation of the titres obtained under our optimum conditions of digestion. All the results were then expressed as percentages of these maximum titres; the spread of the maximum recovery being 99·9 to 100·2 per cent.

Table I

Variation of recovery of nitrogen with amount of sodium sulphate and time of digestion

20 mg. of copper sulphate and 0·35 mg. of selenium in each digestion

Time of digestion	Amounts of Na_2SO_4 (g. to 1 ml. of H_2SO_4)		
	0·08	0·40	1·0
	Recovery of nitrogen		
	%	%	%
30 minutes	91·5	97·0	98·3
1 hour	93·3	98·2	100·0
2 "	94·7	99·0	100·0
3 "	97·0	99·5	100·0
4 "	97·2	99·9	100·2
8 "	100·2	99·6	100·1
14 "	99·8	99·9	99·2

TABLE II

VARIATION OF RECOVERY OF NITROGEN WITH AMOUNT OF SELENIUM AND SODIUM SULPHATE

Amount of Se, mg.	Amount of $CuSO_4$, mg.	Amount of Na_2SO_4 (g. to 1 ml. of H_2SO_4) 0·16 1 hour digestion — Recovery of nitrogen %	0·40 1 hour digestion %	1·0 1 hour digestion %	1·0 30 min. digestion %
nil	nil	—	—	97·9	—
nil	20	—	97·0	99·6	—
0·35	20	95·3	98·2	100·1	98·3
1·75	20	—	99·2	99·9	—
3·5	nil	—	—	99·0	—
3·5	20	97·5	99·0	100·2	99·2
7·0	20	98·2	99·2	—	—
14·0	20	—	99·3	100·1	100·0
28·0	20	—	99·3	99·9	—

TABLE III

VARIATION OF RECOVERY OF NITROGEN WITH AMOUNT OF SELENIUM AND TIME OF DIGESTION

20 mg. of copper sulphate and 1 g. of sodium sulphate in each digestion

Digestion time, hours	Amount of Se, mg. nil — Recovery of nitrogen %	0·35 %	3·5 %
0·5	97·5	98·3	99·2
1	99·6	99·9	100·0
4	100·0	100·1	99·7
14	98·6	99·2	96·4
21	97·8	96·6	94·3

From the tables it would appear that for a digestion time of 1 hour the optimum conditions for complete recovery of nitrogen from 0·1 ml. of serum are the presence of sodium sulphate to the amount of 1·0 g. to 1 ml. of sulphuric acid, and the addition of 3·5 mg. of selenium and 20 mg. copper sulphate as catalysts.

Since lysine is amongst the most refractory of the amino acids in serum proteins, the nitrogen was estimated in a specially pure specimen of lysine mono-hydrochloride under the above conditions. Although the method gave a practically theoretical recovery (99·3 per cent.) this result was only attained when 0·5 mg. samples were used. The recommended method was then compared with the recently described procedure of Hiller, Plazin and Van Slyke,[2] which was shown to be equal to the classical Dumas method. Total nitrogen was estimated in 0·1-ml. amounts of two different sera and the results are shown in Table IV.

TABLE IV

COMPARISON OF PROPOSED METHOD WITH THAT OF HILLER *et al.* (1948)

	Proposed method Nitrogen, mg.	Method of Hiller[2] Nitrogen, mg.
SERUM A		
Mean	1·291 (5)	1·289 (6)
Range	1·287 to 1·296	1·287 to 1·293
Standard deviation	±0·0022	±0·0022
SERUM B		
Mean	1·316 (6)	1·315 (6)
Range	1·313 to 1·321	1·313 to 1·319
Standard deviation	±0·0030	±0·0022

Discussion of results

Certain conclusions can be drawn from these experiments, although as we have confined ourselves to serum proteins, these conclusions may not be applicable to other nitrogen compounds. The results indicate that for maximum recovery of nitrogen, the time of digestion and the amount of sodium sulphate are inversely related; a long period of digestion with a small proportion of the salt will give 100 per cent. recovery of nitrogen just as will a much shorter period with a greater proportion. Other things being equal, it is clearly preferable to use the shortest digestion time consistent with maximum nitrogen recovery and this involves the use of a relatively high proportion of the salt. Too high a proportion, however, may necessitate careful attention to heating so as to avoid loss of acid. Further, the effect of the selenium catalyst is less, at any rate for serum proteins, when the higher proportions of sulphate are used, and hence the use of large amounts of selenium would appear from our results to be unnecessary. It does not appear possible, moreover, to compensate completely for a low proportion of sodium sulphate by increasing the amount of selenium, that is, with a low proportion of the salt, the time of digestion must be longer, and this time is not greatly reduced by increasing the amount of selenium. In protracted digestion, the presence of selenium tends to increase the losses of nitrogen—possibly by increasing the oxidation of ammonia.

Although it was not our purpose to compare the relative efficacy of different catalysts in the micro-Kjeldahl procedure, the statement by Hiller *et al.*[2] that only digestion mixtures containing mercury as catalyst have been found to give nitrogen values as high as those yielded by the Dumas combustion method must be modified in the light of our results. This investigation shows that a method using selenium and copper sulphate catalysts gives results that are in every way comparable with the results obtained by their method using mercury.

Summary

The relationship between time of digestion, amount of sodium sulphate and of selenium as catalyst in the micro-Kjeldahl determination of serum proteins has been investigated.

The optimum conditions for 0·1 ml. of serum are shown to be 3·5 mg. of selenium, 20 mg. of copper sulphate, 1 g. of sodium sulphate and 1 ml. of sulphuric acid for a digestion time of 1 hour.

This method and that of Hiller *et al.*[2] have been compared and shown to be equally accurate.

The above work forms part of a larger investigation on the serum protein levels of infants by B. Levin, Helen M. Mackay (member of the scientific staff of the Medical Research Council) and Catherine A. Neill, with the assistance of V. G. Oberholzer and T. P. Whitehead, who gratefully acknowledge grants from the Medical Research Council.

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Official Appointments

PUBLIC ANALYST APPOINTMENTS

NOTIFICATION of the following appointments has been received from the Ministry of Food since the last record in *The Analyst* (1950, **75**, 393).

Public Analyst	*Appointments*
BUTTON, Donald Frank Harrington ..	Metropolitan Borough of Camberwell.
CAHILL, Terence John (Deputy) ..	Borough of Stockton-on-Tees.
CAHILL, Terence John (Deputy) ..	County Borough of West Hartlepool.
CAHILL, Terence John (Deputy) ..	County Borough of South Shields.
CHAPMAN, Williams Bernard (Deputy) ..	Borough of Gosport.
HARRALL, James Charles (Deputy) ..	County Borough of Bradford.
MINOR, Ronald Gordon (Deputy) ..	Metropolitan Borough of Camberwell.
PIKE, Ernest Richard (Deputy) ..	County Borough of Leicester.
RYMER, Thomas Edward (Deputy) ..	County Borough of Croydon.
WOOD, Eric Charles	County Borough of Ipswich.

OFFICIAL AGRICULTURAL ANALYST APPOINTMENTS

NOTIFICATION of the following appointments has been received from the Ministry of Agriculture and Fisheries since the last record in *The Analyst* (1950, **75**, 393).

Agricultural Analyst	*Appointments*
CAHILL, Terence John (Deputy) ..	County of Cumberland.
CAHILL, Terence John (Deputy) ..	County Borough of West Hartlepool.
CAHILL, Terence John (Deputy) ..	County Borough of South Shields.
CHAPMAN, Williams Bernard (Deputy) ..	County of Isle of Wight.
RYMER, Thomas Edward (Deputy) ..	County of Surrey.
RYMER, Thomas Edward (Deputy) ..	County Borough of Croydon.
VOELCKER, Eric	County of Huntingdon.
WILLIAMS, Albert Lester	County of Isle of Wight.
WOOD, Eric Charles	County Borough of Ipswich.

Ministry of Food

STATUTORY INSTRUMENTS*

1950—No. 1313. The Flour (Amendment No. 3) Order, 1950. Price 1d.

This Order, which amends the Flour Order, 1947, as amended, provides for the rate of extraction of national flour to be reduced from 85 per cent. to 81 per cent. on August 27th, 1950, and of "W" flour from over 85 per cent. to over 81 per cent. from the same date.

CIRCULAR MF 15/50

This circular, dated 16th August, 1950, contains the information that, as a result of the decision in the King's Bench Division in the case of Kat v. Diment, the Ministry of Food Code of Practice relating to Vinegar and Solution of Acetic Acid, printed at p. 55 of "The advertising, abelling & Composition of Food" (H.M. Stationery Office, 1949) has been withdrawn.

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

Reviews

RADIOACTIVE INDICATORS. THEIR APPLICATION IN BIOCHEMISTRY, ANIMAL PHYSIOLOGY AND PATHOLOGY. By GEORGE HEVESY. Pp. xvi + 556. London: Interscience Publishers Ltd. 1948. Price 60s. net.

The possibility of using radioactive isotopes as indicators in biological systems was first demonstrated by Professor Hevesy more than 25 years ago. In a celebrated paper published in the *Biochemical Journal* in 1923, he described "the absorption and translocation" by plants of thorium B, a naturally-occurring radioactive isotope of lead. Although no further tracer experiments could be undertaken until after the discovery of deuterium and the invention of the cyclotron 10 years later, the concepts introduced by Hevesy in his original investigation have been accepted as the basis of modern tracer techniques. In the words of Professor Rittenberg, Hevesy "was not only one of the fathers of the isotope technique, but also the attending gynaecologist." His contributions to the subject have been recognised by the award of a Nobel Prize in 1943 and a Faraday Medal a few weeks ago. Such is the reputation of the author of this monumental volume on the application of radioactive isotopes in animal physiology, pathology and biochemistry.

Although applications in plant physiology and all references to stable isotopes have been omitted, this book provides the most comprehensive review of the isotope technique that has yet appeared. While writing the book, Hevesy was resident in turn at the Universities of Copenhagen, Stockholm and California, and he is thus able to appraise both European and American work. Although the book was printed in the United States, the European system of indicating atomic mass by a raised prefix (*e.g.*, ^{14}C) rather than a raised suffix has been adopted.

The first three chapters describe the production and measurement of radioactive isotopes and include a list of the isotopes that were available from the United States Atomic Energy Commission in September, 1947. In future editions it should be mentioned that most of these isotopes can be supplied by the British Atomic Energy Research Establishment.

The applications of isotopes in chemical analysis are described in one short chapter. Subsequent chapters deal with the measurement of the absorption, distribution and excretion of elements, phase boundary permeability and turnover-time.

Although for certain specialised aspects of the subject the treatment is neither as up-to-date nor as detailed as that given in special monographs, such as Calvin's "Isotopic Carbon" (see *Analyst*, 1950, **75**, 113), the volume is fully up to the standard to be expected from Professor Hevesy. It should be in the hands of all persons who are either proposing to use radioactive isotopes in biological studies or are interested in the important results obtained by their means.

J. E. PAGE

HIGH POLYMERIC CHEMISTRY. By W. S. PENN, B.Sc. Pp. xvi + 487. London: Chapman & Hall Ltd. 1949. Price 36s. net.

The purpose of this book is to describe the theories that govern the formation and properties of high polymers, and to stress throughout the chemical point of view. Although not neglected, less emphasis is paid to the practical aspects of the question. The subjects dealt with include the mechanisms whereby large polymer molecules are formed and methods of following the reactions involved, hydrocarbon substituted polymers and vinyl polymers, polydienes from hydrocarbons and halogenated hydrocarbons, condensation resins, resins from natural products, proteins and thermosetting resins. There is a useful chapter on the preparation of monomers.

Errors and spelling mistakes and misprints in formulae are relatively few and in most cases the true meaning is obvious from the text. There are, however, a few points to which attention should be drawn.

It might be useful in future editions to give, throughout the book, the original sources of information about the properties of the polymers that are shown in tabular form. On p. 77 it should be pointed out that the determination of the iodine number by the Wijs' method involves the titration of the iodine liberated by a known volume of the Wijs' reagent itself. The section on "Analytical Schemes" on p. 95 might be improved considerably. It is suggested on p. 96 that by following the procedure given in 1½ pages of text it should be possible to ascertain exactly the nature of any high polymeric material; this statement is quite misleading. On p. 296 it is difficult to see exactly how conductivity cells are to be used in the determination of viscosity.

The author of this book hopes that the treatise may be accepted as a standard textbook, and although it may be doubtful whether this desire will be realised, there is no doubt that he has brought together a very great deal of information about the various theories underlying the structure and formation of polymers.

To young chemists entering this field the book is likely to be extremely useful because of the comprehensive character of the references to original papers on the subject. J. HASLAM

A CHEMISTRY OF PLASTICS AND HIGH POLYMERS. By PATRICK D. RITCHIE. Pp. viii + 288. London: Cleaver-Hume Press Ltd. New York: Interscience Publishers Inc. 1949. Price 25s.

This book is concerned primarily with the organic chemistry of high polymers and it has very little to tell of their applications, testing or analysis. Because of the wide differences in character between high polymers the book falls into distinct sections, dealing with (*a*) synthetic plastics, (*b*) natural products such as proteins and cellulose, and (*c*) a number of inorganic materials such as graphite, and zeolites and other silicates. There is also a short review of the relation between the structures of high polymers and their physical properties. The identity of the various materials and the reactions by which they are prepared are clearly described. The book will provide a readable and useful guide to degree students, for whom it was chiefly designed. G. H. WYATT

Publications Received

THE PHARMACOPOEIA OF THE UNITED STATES OF AMERICA. Pp. lv + 1067. New York: Mack Publishing Co. 1950. Price $9.00.

CELLULOSE ACETATE PLASTICS. By VIVIAN STANNETT. Pp. xxiv + 325. London: Temple Press, Ltd. 1950. Price 30s. net.

PHYSICO-CHEMICAL CONSTANTS OF PURE ORGANIC COMPOUNDS. By J. TIMMERMANS. Pp. viii + 693. New York and Amsterdam: Elsevier Publishing Company, Inc. London: Cleaver-Hume Press, Ltd. 1950. Price 95s. net.

THE CHEMISTRY OF INDUSTRIAL TOXICOLOGY. By H. B. ELKINS. Pp. ix + 406. London: Chapman & Hall, Ltd. New York: J. Wiley & Sons, Ltd. 1950. Price 44s.; $5.50.

THE PHARMACEUTICAL INDUSTRY IN GERMANY DURING THE PERIOD 1939–1945. British Intelligence Objectives Sub-Committee Surveys Report No. 24. Pp. 120. London: H.M. Stationery Office. 1950. Price 3s. 6d.

MELTING AND SOLIDIFICATION OF FATS AND FATTY ACIDS. By A. E. BAILEY. Pp. xiv + 357. New York and London: Interscience Publishers, Inc. 1950. Price 56s.

INTRODUCTION TO SEMI-MICRO QUALITATIVE CHEMICAL ANALYSIS. Revised Edition. By L. H. CURTMAN. Pp. vii + 391. London and New York: Macmillan & Co., Ltd. 1950. Price 28s. 6d.

A DICTIONARY OF DAIRYING. By J. G. DAVIS, D.Sc., Ph.D. (Lond.), F.R.I.C. Pp. xx + 856. London: Leonard Hill, Ltd. 1950. Price 37s. 6d.

QUANTITATIVE ULTRAMICROANALYSIS. By P. L. KIRK, Ph.D. Pp. vii + 310. London: Chapman & Hall, Ltd. New York: J. Wiley & Sons, Ltd. 1950. Price 40s.; $5.00.

BIOLOGICAL STANDARDISATION. By J. H. BURN. London: Oxford University Press. 1950. Price 35s.

ANLEITUNG ZUR DARSTELLUNG ORGANISCHER PRÄPARATE MIT KLEINEN SUBSTANZMENGEN. By HANS LIEB and WOLFGANG SCHÖNIGER. Pp. xi + 158. Vienna: Springer-Verlag. 1950. Price 18s.

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THE ANALYST

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