Some Experiments with the Spekker Photo-electric Absorptiometer and Fluorimeter

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Mr. Isbell[1] has given a very clear picture of the difficulties encountered by the manufacturer when attempting to mass produce an absorptiometer and fluorimeter. We think that Messrs. Hilger & Watts deserve praise for the ingenuity and perseverance with which they are attempting to overcome these difficulties. Our task now is to describe how the various instruments under review stand up to the stress of analytical practice.

Previous work on the Spekker absorptiometer has shown it to give quite satisfactory results in the photo-electric estimation of intensity of colour. Provided that due precautions are taken to avoid turbidity in the solutions examined, the precision obtainable is superior to that experienced with the most careful visual colorimetry. For example, in eighty consecutive assays of nicotinic acid in different foodstuffs, using the original model of the "Spekker", we obtained in our duplicates an average percentage standard deviation (coefficient of variation) for a single assay of 0·88, compared with 1·3 per cent. obtained by Kodicek[2] in fifteen visual estimations of pure nicotinic acid solutions and 3·4 per cent. in sixteen estimations of nicotinamide, using the Zeiss step-photometer but otherwise the same conditions, including the same aromatic amine, as ourselves. Taking into consideration the other sources of error in the assays, we think that in the actual measurement of the colour intensity a difference of 1 per cent. with the "Spekker" would be at least as readily detectable as a difference of 2 to 5 per cent. in visual colorimetry.

Recently we have had the opportunity of testing in our laboratory a prototype of the new model of the Spekker absorptiometer. The larger drum and two-speed control, plus the more rapid lamp-house shutter, should improve the speed and accuracy of the readings. We have found them of much value when examining unstable colours such as occur in nicotinic acid assays. The enlarged holder to take three cuvettes facilitates the comparison of the unknown with two standards, one stronger and the other weaker than the unknown. The increased provision for liquid filters allows greater specificity to be obtained, but it would be an advantage if the filter holders allowed more flexibility in the size of the filters used.

Deviations from linearity in fluorimetry

Turning next to fluorimetry, this presents special difficulties which can cause serious deviations from linearity—

(*a*) The light energy measured is only 1 or 2 per cent. of the incident light energy,[3] hence minute amounts of stray light produce proportionately large effects.

(*b*) Impurities present may cause "quenching" of the fluorescence.[4] The resulting deviations from linearity can be minimised by using more dilute solutions,[5] but the instrumental blank then forms a larger fraction of the total reading and so increases the deviation from linearity, unless due allowance is made for the blank.

(*c*) The necessity for a continuously operating light source (as compared with intermittent light in absorptiometry) generates heat, which may—

(i) penetrate to the filters and affect their transmission;

(ii) reach the liquid in the cuvette and affect its emission of energy.

In the new Spekker fluorimeter, heat from the ultra-violet lamp, which sometimes gave trouble in the old "Spekker," is dissipated by means of improved ventilation, and the eyes of the operator are more effectively protected from the ultra-violet light. The shutter, which

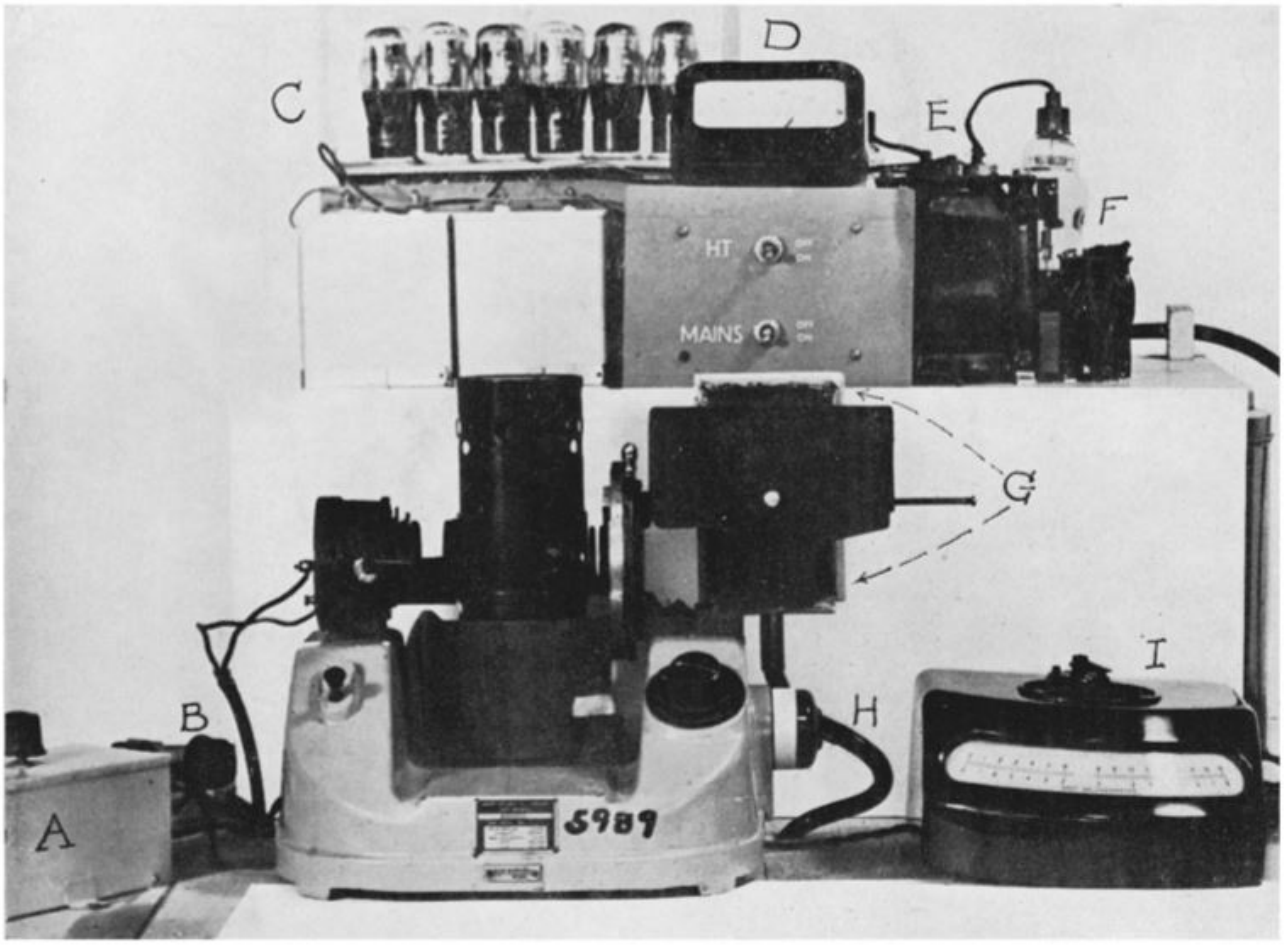
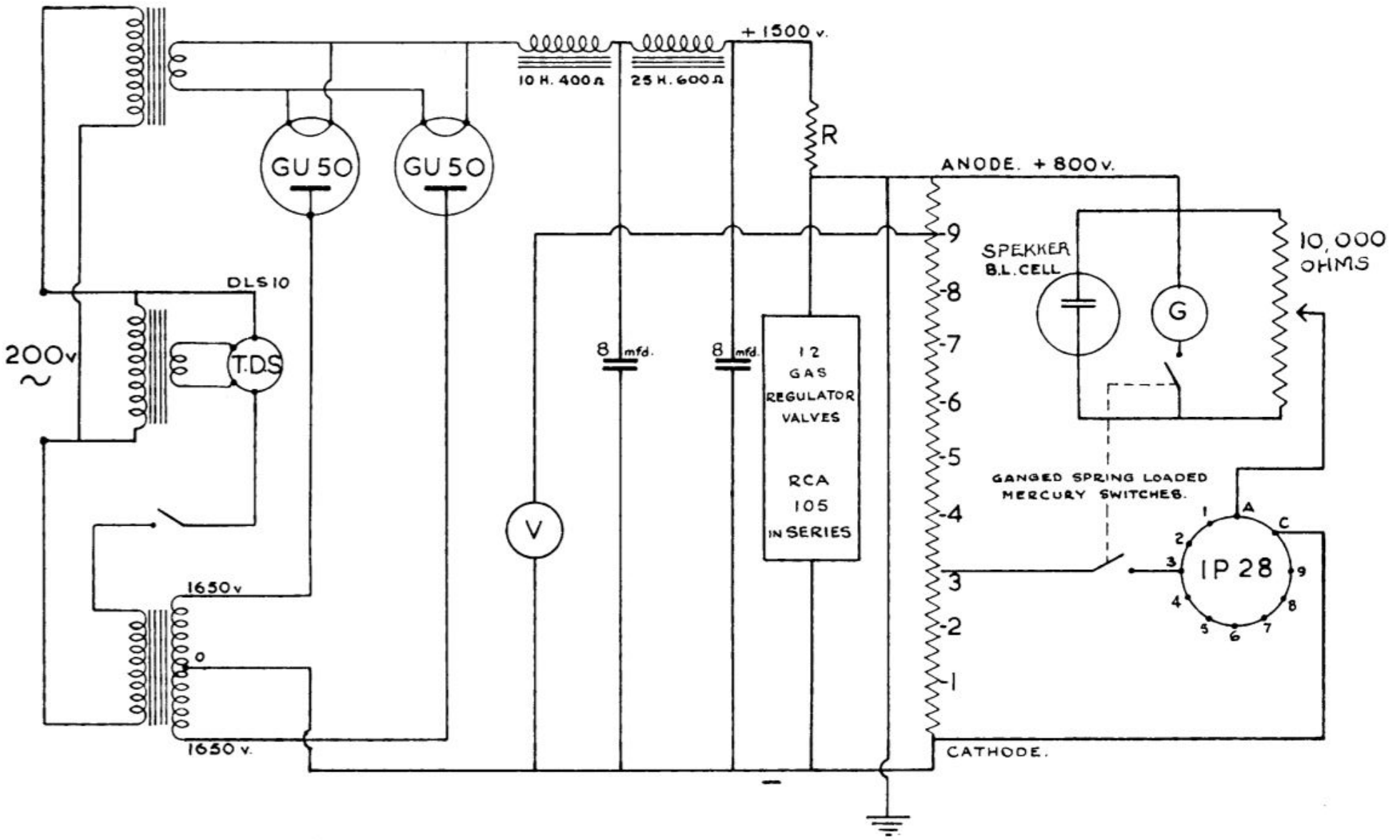


Fig. 1. Illustration of electron multiplier with power pack as applied to Spekker fluorimeter
A. Ganged spring-loaded mercury switches
B. Galvanometer shunt
C. Power pack (12 stabiliser valves in two rows of 6)
D. Dynode voltage indicator
E. High tension transformer
F. High tension filament transformer
G. Multiplier housing-adaptor machined to fit original B.L. cell cavity
H. Screened multiway polythene cable
I. Cambridge spot galvanometer, used as null point or deflection indicator
C, D, E, and F are normally kept under working bench remote from operator



IP 28 MULTIPLIER & POWER SUPPLY CIRCUIT.

Fig. 2. Wiring diagram of electron multiplier with power pack

in the old instrument could become stiff and uncomfortably hot to handle, is considerably improved, but because the galvanometer remains alive it must be guarded against strain when opening the cuvette housing and thus exposing the photo-cells to light. We are glad to note that since our report to the makers on the prototype of the new fluorimeter, steps have been taken to meet this difficulty by adding to the instrument a guard which automatically covers the photo-cells from light when the housing is opened. They have also accepted our suggestion to include more calibrations in the right-hand transmission scale, especially between 70 and 90, thus permitting readings of reciprocal of antilog density to be taken directly, and saving two steps in calculations when allowance is being made for the instrumental blank as described below (p. 625). The linear calibrations in the left-hand density scale are certainly easier to evaluate once one has been accustomed to the "upside-down" scale. Space is available for only one primary filter on the *left*-hand side of the drum, compared with two in the old instrument. On the right-hand side of the drum, space might be contrived for further filters, including a liquid filter for the primary beam (*e.g.*, copper sulphate solution to remove red rays transmitted by Wood's glass). More space also should be available for secondary filters, to meet the demand for narrower band filters, which will increase as their advantages become more widely appreciated.

For analysts interested in riboflavine estimations the most important improvement in the new "Spekker" is probably the marked increase in sensitivity obtained by the use of two large barrier-layer photo-cells. The increase in the photo-receptive area utilised does, however, raise a certain difficulty in that stray light (*e.g.*, reflection of the primary beam from the lid or walls of the cuvette) is afforded more opportunity of reaching the photo-cell. This difficulty can to some extent be overcome by using a mask to cut down the top of the photo-receptive area, and experiments in the use of such masks are now in progress. Even if the primary beam is passed through Wood's glass and highly selective secondary filters are employed, this source of error can exert an appreciable effect in fluorimetric assays of aneurine and riboflavine, especially the latter. Moreover, some barrier layer cells are not satisfactory for measuring very feeble light because of "residual current." The "Spekker" attempts to avoid this by bringing the photo-cells as close as possible to the cuvette, which of course reduces the amount of space available for secondary filters. All these difficulties can be overcome by replacing the barrier-layer photo-cell by a photo-multiplier, obtaining an immense increase in sensitivity which permits the use of a much smaller photo-receptive area at sufficient distance from the cuvette to provide ample space for a variety of secondary filters. Dr. Menzies, of Messrs. Hilger & Watts, has taken much interest in experiments we have been making on the application of photo-tubes to the Spekker fluorimeter, and is considering the possibility of arranging that such photo-tubes can be employed with the new "Spekker."

Experiments with prototype fluorimeter

Before describing our experiments with photo-multipliers we should like to present some data obtained with a prototype of the new Spekker fluorimeter, which we were able to test in our laboratories last summer. This prototype was successfully employed in fluorimetric assays of various substances, including aneurine and riboflavine, but we shall refer mainly to the latter vitamin, which presents the greater difficulties. The best method of testing a fluorimeter is to calibrate it with the given fluorescent substance over a sufficiently wide range of concentrations to cover any measurements that may have to be made. Table I summarises data obtained in the calibration of the new "Spekker" with riboflavine solutions matched against a fluorescein standard (referred to as F/1 in the Tables) containing 1 μg. per ml. in phosphate buffer solution of pH 6. The aim is to obtain linearity of response, as shown by a constant ratio between fluorescence intensity and concentration of fluorescent substances. However, in the Spekker instrument, the use of a balanced circuit, whilst bringing certain marked advantages, does mean that the instrumental blank is included in the "density" measured. This involves a departure from linearity becoming more and more marked as more dilute solutions are examined. To overcome this difficulty we took the antilog density for a given concentration and deducted from it the instrumental blank determined on the cuvette filled with the phosphate buffer solution only. In this particular experiment the mean density given by this blank against the fluorescein standard F/1 was 0·869. The antilog of this was 7·396 and the reciprocal of this (0·135) was deducted from each observed fluorescence to obtain the net fluorescence. For the more dilute solutions

the zero was set on the riboflavine because its fluorescence was weaker than that of the fluorescein standard, and the reciprocal of the antilog density was, therefore, employed to calculate the net fluorescence. This net fluorescence when divided by the concentrations gave a fluorescence/concentration ratio which (apart from the concentration quenching with the three strongest solutions) remained remarkably constant between 2·74 and 2·75 over the

TABLE I

CALIBRATION OF NEW SPEKKER FLUORIMETER WITH RIBOFLAVINE SOLUTIONS AGAINST FLUORESCEIN STANDARD F/1 (1 μG./ML.)

Riboflavine μg./ml.	Mean "density" against F/1	Antilog density (AD)	$\frac{1}{AD}$ = RAD	RAD − blank = net fluorescence	$\frac{\text{Fluorescence}}{\text{Concentration}}$
F/1 at zero					
4	0·833	6·806		6·67	1·67
2	0·634	4·305		4·17	2·09
1	0·417	2·612		2·48	2·48
Riboflavine at zero					
¼	0·086	1·219	0·821	0·686	2·74
⅛	0·320	2·089	0·479	0·344	2·75
1/16	0·514	3·262	0·307	0·172	2·75
1/32	0·656	4·529	0·221	0·086	2·75

range from ¼ to 1/32 μg. per ml. The linearity thus obtained compared favourably with that found by Umberger and LaMer,[6] who used fluorescein solutions, the fluorescence of which was similar to that of our weakest riboflavine solutions, in a fluorimeter that they claimed to be more sensitive than any so far described in the literature.

VARIATION IN INSTRUMENTAL BLANKS

When the Spekker fluorimeter is calibrated in the manner we have just described, the degree of linearity obtained depends largely on the accuracy with which the instrumental blank can be determined. Table II shows how this blank can vary widely with different cuvettes, and with the same cuvette used under different conditions. In a series of five cuvettes supplied with ground glass lids the instrumental blank in riboflavine estimations,

TABLE II

INSTRUMENTAL BLANKS IN RIBOFLAVINE ESTIMATIONS WITH NEW SPEKKER FLUORIMETER

Primary filter	Blank as % of F/1, using cuvettes with lids of: Ground glass	Clear glass
Wood's	20, 18, 19, 18	9, 9, 15
	16, 18, 22, 23	16, 12, 14
	20, 18, 19	
	22,* 26,† 18**	11,†† 19§
Wood's + copper sulphate	13,* 15†	
Wood's + H503	13**	10,†† 8§
Wood's + large H503		8,†† 6§

* These results obtained with the same cuvette.
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whilst varying somewhat between different estimations, was on the whole much higher than in a series of three cuvettes with clear glass lids. This was probably due to the reflection from the ground glass lids of red rays which were transmitted by the secondary filters used in riboflavine estimations, because when the primary Wood's glass filter was supplemented by a liquid copper sulphate filter cutting out the red rays, the blanks were much reduced. When cuvettes with clear glass lids were used, the inclusion of the H503 filter to exclude heat rays produced on the whole rather smaller reductions in the blanks. Still smaller blanks are obtained by using black glass lids.

Application of photo-multipliers to spekker fluorimeter

In some estimations, *e.g.*, that of riboflavine, it is desirable to employ rather low concentrations of fluorescent substances. These give with barrier-layer photo-cells insufficient outputs to provide satisfactory galvanometer responses. If the latter are amplified optically, by increasing the distance between the light and the mirror, serious difficulties can arise because of vibrational effects. Vacuum-type photo-cells and amplifiers with high grid resistance tend to be rather temperamental under normal working conditions. These difficulties are obviated by the use of photo-multipliers, with their more easily stabilised high voltage supply. The enormous increase in sensitivity obtained with these photo-multipliers opens out such possibilities in fluorimetry that their widespread employment may eventually be expected. During the past two years we have been experimenting with a variety of photo-multipliers, both American and British types. Fig. 1 shows our set-up as applied to an old Spekker fluorimeter originally purchased in 1941. In this set-up, which has been in regular use in our laboratory for nearly a year, the photo-multiplier is mounted in a light-tight box attached to the back of the cuvette housing. The fluorescent light enters this box through an aperture about 1 cm. in diameter, which is in the position formerly occupied by the barrier-layer photo-cell. On striking the photo-receptive area on the cathode it produces a current which is amplified about 45,000-fold, using the present dynode voltage of 80 volts per stage. Provision has been made for operating at 125 volts per stage, which will increase the current amplification to about 800,000. The enormous increase in sensitivity thus obtained permits the use of a stable spot galvanometer in place of the mirror galvanometer previously employed, and thus obviates any need for anti-vibration devices. The dynode voltage per stage is measured by a voltmeter permanently in circuit for check purposes and has been found to remain remarkably constant. Fig. 2 shows the wiring diagram.

A difficulty in multiplier use is effective regulation of the high voltages required. We have employed a series of twelve R.C.A. 105 gas diodes with a potential of 1260 volts across them. Constancy to a quarter of a volt per stage is maintained for main input changes of 180 to 230 volts A.C., a control of potential sufficiently close to provide satisfactory results.

These gas diodes used as stabilisers provided a very simple solution to the problem of dynode voltage regulation and, although relatively cumbersome compared with electronic methods, have proved remarkably stable during over a year of constant usage.

To facilitate the measurement of very faint fluorescence we adopted the expedient of "backing off" the blank deflection by means of the left-hand photo-cell of the "Spekker," applying its output in reverse and returning the galvanometer to zero by optical light control with the iris diaphragm. This enabled us to measure the fluorescence of very dilute quinine and fluorescein solutions, down to a thousandth of a microgram per millilitre, which is considerably lower than has been previously reported.

Table III gives some calibration data obtained with this old "Spekker" plus photo-multiplier, and shows that the degree of linearity, as measured by the fluorescence/concentration ratio, compared favourably with that given by the new "Spekker."

Table III

Calibration of old spekker fluorimeter plus photo-multiplier against fluorescein standard F/1 (1 μg./ml.)

Riboflavine μg./ml.	Mean "density" against F/1	Antilog density (AD)	$\frac{1}{AD}$ = RAD	RAD − blank = net fluorescence	$\frac{\text{Fluorescence}}{\text{Concentration}}$
F/1 at zero					
1	0·258	1·809		1·77	1·77
½	0·004	1·009		0·968	1·94
Riboflavine at zero					
¼	0·250	1·778	0·562	0·521	2·08
⅛	0·520	3·311	0·302	0·261	2·09
1/16	0·760	5·754	0·174	0·133	2·13
1/32	0·980	9·55	0·105	0·064	2·05

Comparison of fluorimeters

In the comparison of fluorimeters for analytical purposes two main factors must be considered—

(*a*) sensitivity;
(*b*) instrumental blank.

Table IV gives data on these factors for the old "Spekker," the new "Spekker" and the old "Spekker" plus R.C.A. I.P.28 photo-multiplier, when used in aneurine and riboflavine estimations. It will be seen that the new "Spekker" has 5 to 7 times the sensitivity of the old "Spekker," and that the addition of the photo-multiplier to the old one increases its sensitivity 63-fold in riboflavine estimations, and 75-fold in aneurine estimations. The instrumental blanks are all only 1 to 3 per cent. of the quinine and fluorescein standards, except in riboflavine solutions with the new "Spekker," when they reach 10 per cent. or more. This is due largely to reflection from the cuvette lids of red light in the incident beam, because when the clear glass lids are replaced by black glass lids, absorbing this light, the instrumental blank in riboflavine estimations is considerably reduced.

TABLE IV

COMPARISON OF FLUORIMETERS

	Relative sensitivity for estimating		Instrumental blanks as % of	
	Aneurine	Riboflavine	Quinine standard Q/1	Fluorescein standard F/1
Old "Spekker"	1	1	2	3
New "	5	7	1–2	10
Old " plus I.P.8 photo-multiplier	75	63	2–3	1½–2

NOTE—

All estimations made using Tinsley galvanometer and normal set-up of filters, etc. Photo-multiplier used at only 78 volts per stage.

Later results indicate that the R.C.A. I.P.21 photo-multiplier has definite advantages over the I.P.28 photo-multiplier in riboflavine estimations, as we hope to show in a further publication.

The instrumental blank is due mainly to unwanted reflection from cuvette lid and walls of improperly filtered light (primary or fluorescent or both). Improvements will therefore come from—

(*a*) reducing the photo-cell aperture to minimise the amount of unwanted reflection that reaches the photo-receptive area; and

(*b*) better control of filtering properties both in the primary beam and in the fluorescent light.

Different instruments may vary in the relative importance of (*a*) to (*b*), and the instrument will give different results when the exciting light wavelength or the fluorescent light band is changed.

The greatest improvements in regard to blanks will probably come from the use of—

(*a*) a copper sulphate filter in the primary beam to eliminate red rays when using red-sensitive detectors such as barrier-layer cells;

(*b*) narrower cut secondary filters transmitting practically none of the primary light used;

(*c*) photo-multiplier tubes, giving extra sensitivity which will allow of (*b*) and of small apertures.

APPLICATION OF FLUORESCENCE SPECTRA

We have recently been employing fluorescence spectra to explore the disturbing factors responsible for these instrumental blanks. Light from the Spekker ultra-violet lamp, after being filtered through Wood's glass, was passed into the cuvette containing the liquid under examination and the radiation from the cuvette was taken at right-angles into a Hilger wave-length spectrometer with camera attachment. The fluorescence spectra thus obtained indicated that the light from the instrumental blank is most intense in the spectral region corresponding to the fluorescein fluorescence used in riboflavine assays, and much weaker in the spectral region corresponding to the quinine fluorescence used in aneurine assays. Thus an explanation is provided for the higher blanks observed with the "Spekker" in riboflavine assays. These high blanks can be considerably reduced by altering the cuvette lids, and especially by cutting down the size of the photo-sensitive surface employed to detect the fluorescence, in which the use of photo-multipliers will be very important.

The spectrometer has also been used to investigate the nature of the incident beam obtained when Wood's glass is used as primary filter. It was found that an appreciable amount of visible light was passing through the Wood's filter. Since this provided the possibility of obtaining readings too high in fluorimetric assays, especially of riboflavine, experiments have been carried out on the replacement of the Wood's filter by Wratten **39** and **47** filters, in the presence of a **H503** heat-resiting filter. These enabled us to obtain fluorimetric results in much closer agreement with microbiological assays on the same samples, containing a good deal of interfering substances. Full details of these experiments will be published later.

Spectroscopic methods can be used to check the efficiency of various analytical procedures and should lead to further advances in the science of fluorimetry.

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